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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this research project is to study the role of neuronal sodium channels in mechanisms of neuronal injury, neurodegeneration and neuroprotection. The primary objective of this research project is to characterize the expression, and study the functional significance, of neuronal sodium channels during the process of injury and recovery. Also, the effects of sodium channel blockade (using antisense and channel blockers) on gene expression and neurodegeneration will be studied.

The progress during our 2nd year of this project was significant, yielding several important and critical discoveries providing a greater understanding of the injury/neurodegeneration process, and the importance of sodium gated channels in brain trauma. Three new discoveries were 1) our finding that during the brain injury process there appears to be a selective alteration in the rBI brain sodium channel with little involvement of rBIII, PNI or the PN3 channels, 2) our observations obtained from the in situ hybridization experiments that during brain injury supposedly uninjured brain tissue is indeed compromised (at least on the level of the ion channel) altered functionally as evidenced by our topographic mapping EEG studies (conducted independent of this research proposal), and 3) that sodium channel blockade with the novel drug RS100642 is highly neuroprotective and has potent therapeutic anti-seizure actions post brain trauma. We are currently developing primers for additional sodium channels and sodium-calcium exchange proteins and plan to study their expression post injury as well as complete the *in situ* hybridization and ASO experiments during the final year of this project.

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INTRODUCTION

Severe disruptions in ionic dynamics of the depolarizing neuron have long been recognized as possible mediators of excitotoxicity. In neurodegeneration leading to neuronal cell death, injury-induced imbalances in intracellular calcium ($[Ca^{++}]i$) have received considerable attention, leading many, including our own laboratory, to propose the "calcium mechanism" of neuronal injury and neurodegeneration. However, of possibly equal consequence to the responsiveness of an injured neuron is the state of the Na^{+}-Ca^{++} exchanger, and the influence of altered sodium dynamics to promote calcium overload, presynaptic membrane depolarization, and excitation. The pathophysiological importance of neuronal sodium channels to membrane stabilization has been recognized and studied in epilepsy, and more recently in peripheral neuropathy and other neurodegenerative conditions. The results of these studies have led to the cloning, sequencing and physiological characterization of at least four neuronal sodium channels. However, despite these discoveries and the exciting prospect of protecting neurons against excitotoxic insults with the development of novel sodium channel blocking drugs, the role of sodium channels in cell death mechanisms and neuroprotection has received relatively limited attention. It is becoming increasingly apparent that blockade of neuronal sodium channels may prevent, or at least attenuate, neurodegeneration and offer an exciting therapeutic approach for the treatment CNS injury. Therefore, the primary objective of this research proposal is to characterize the molecular expression and determine the functional significance of the respective neuronal sodium channel genes relative to the development and recovery mechanisms of the injury process. A second objective is to study the effects of sodium channel blockade on the molecular and cellular consequences of neuronal injury, and its influence to improve recovery and repair mechanisms. These molecular, cellular and pharmacological studies directly address the involvement of sodium channel mechanisms in neuronal injury and will provide significant insights to the role of brain sodium channels in injury-induced neurodegeneration, and the potential therapeutic consequences of sodium channel blockade (and/or decreased expression) on the neurodegeneration process.

BODY

I. Original "STATEMENT OF WORK"

Year 1:

Since we have successfully characterized and standardized the RT/PCR methods and determined that at least four of the neuronal sodium channels are expressed in normal rat brain, the focus of the first year will be to initiate experiments aimed at establishing the functional significance of each of the respective sodium channel genes to anoxia/ischemia-mediated neurodegeneration. This will be accomplished using antisense oligonucleotides (ASOs) and quantitative measures of neurodegeneration in both the *in vitro* neuronal culture model of hypoxia/hypoglycemia (H/H) injury (completed first) and the *in vivo* 72 hr recovery middle cerebral artery occlusion (MCAo) model. Also, *in vitro* pharmacological experiments (some of which are already underway) will be completed evaluating the neuroprotective properties of the sodium channel blocker mexiletine in three neuronal culture models of neurodegeneration (H/H, veratridine and glutamate). In parallel experiments, an *in vivo* dose-response experiment will be initiated in the rat MCAo 24 hr recovery model to determine the neuroprotective potency and efficacy of systemic mexiletine.

Year 2:

The functional studies using ASOs will be completed. Anticipating that these experiments will reveal which (if not all) of the sodium channels is functionally involved with developing neurodegenerative processes, we will begin comprehensive *in vitro* (H/H) and *in vivo* (72 hr MCAo) RT/PCR time-course experiments and examine the influence of the injury state on the expression of the sodium channel genes. We will also initiate *in situ* RT/PCR experiments to characterize the regional changes in the localization of the expressed sodium channels in normal and injured rat brain tissue.

Year 3:

The RT/PCR time-course and the *in situ* RT/PCR will be completed. Also, the experiments examining the effect of sodium channel blockade with mexiletine on injury related changes in sodium channel gene expression in the 72 hr MCAo model will be completed.

II. General Summary

Year two of our research project was highly successful and we believe that we met, and in some instances exceeded, our objective for the proposed research plan. With the advent of our complete and successful transition into the new WRAIR research facilities, our research efforts were able to progress with minimal delays. There were no major administrative problems during FY'02. Unexpected research problems encountered during the course of the year were dealt with aggressively and with complete success and are defined below in detail. We do not believe at this time that these setbacks will effect our meeting our stated objectives for the third and final year of the project.

III. Administrative Problems/Accomplishments for FY'02

No serious administrative problems were encountered. All procurements proceeded on schedule and without delay. Re-budgeting was approved for the purchase of an uninterrupted power supply (UPS) for the TaqMan and we no longer risk losing valuable and expensive data during building 503 power failures. Also, re-budgeting was approved for the purchase of an additional surgical microscope which has facilitated our *in vivo* brain injury research and has allowed us to make significant, additional progress on the gene expression and neuroprotection studies. Importantly, these additional procurements were made without any additional expense to the MRMC.

IV. Research Problems/Accomplishments for FY'02

1. Description of Problems encountered:

During the second funding period of the grant following technical problems were encountered:

1. The major problem encountered in the second year of funding was with *in vitro* antisense oligonucleotide (ASO) studies. We developed and synthesized (commercially) the ASO for all the four sodium channel genes during the first year. Studies using these ASOs were initiated using primary cultures of forebrain neurons and hypoxia/hypoglycemia (H/H) and veratridine toxicity models. Increasing doses (0.05-5.0 μM) of these ASOs were used to pre-treat cells (24-4 hr pre-treatment) prior to injury. No significant neuroprotection was observed using rBIII, PN1 or PN3 ASO (at any concentrations or pre-treatment time intervals studied) in both the injury models. This is consistent with our in vivo gene expression experiments where little significant change in these genes has been seen following brain injury. On the other hand, rBI ASO provided a limited neuroprotection when treated 24 hr before the insult at 1-5 μM concentrations, a result consistent with our observed changes in the rBI gene expression profile following brain injury. However, the rBI ASO treatment also produced mild cytotoxicity and increased protease activity that resulted in cell detachment from the culture wells. This situation was unique for only the rBI ASO, as the other three sodium channel gene ASOs did not

produce any toxicity and had no effect on protease activity. This problem also increased the variability of our results.

To solve this problem we tried co-treating the neurons with one of the three different protease inhibitors. Lower concentrations of these inhibitors had no significant effect on the protease activity and higher concentrations of inhibitors were slightly effective (however, these inhibitors alone provided 5-10% neuroprotection, complicating the outcome of our results). **This problem still remains unsolved and we are now attempting got to develop another set of ASO for the rBI gene. Alternatively, we are also testing the original rBI ASO in our *in vivo* model where cell detachment from culture plates is not a problem.**

2. During the end of FY'01 we initiated the *in situ* RT/PCR studies continued these experiments into FY'02. We experienced several technical difficulties with this phase of our research including obtaining good quality thin (10 μm) **frozen sections** to standardize the RT/PCR conditions (frozen sections were desired so that we could generate tissue from the same injured brains used for TTC staining of infarct volume). With assistance from our Division of Pathology, this methodology was successfully mastered by our staff and studies were initiated. A second problem was encountered when we moved to the MCAo injured rat brain. Since our injured rat brains have very large areas of tissue infarction on the ipsilateral hemisphere cutting intact frozen sections (10 μm thick) of injured brain became almost impossible, with a great deal of tissue lost from the injured side of the brain during cutting and mounting of sections. On occasion where successful sections were obtained, we determined that the very fragile area of injured tissue was either further damaged, or lost, during subsequent RT/PCR steps. After many consultations with Pathology experts in the field, we decided to undertake our *in situ* hybridization studies using fixed tissues instead of RT/PCR studies using frozen sections requiring the need to include separate sets of animals for this purpose only.

   Since the quantitative RT/PCR method requires frozen sections to provide it was necessary to switch to an different *in situ* hybridization technique to visualize the mRNA. Fixed sections are routinely used for *in situ* hybridization studies and both RT/PCR and *in situ* hybridization essentially provide the same end result. Therefore, with the help of Dr. Eric Yu (from NMRC; an expert in this field), **we were successful in obtaining good quality *in situ* hybridization data for β-actin mRNA in our trial study.** This effort has now been extended to the rBI sodium channel gene and we are obtaining excellent and exciting *in situ* hybridization results. (see enclosed abstract submitted to the Society for Neuroscience annual meeting). Studies are currently under progress to study the changes in other sodium channel genes (rBIII, PN1 and PN3) following MCAo injury using *in situ* hybridization technique.

2. Description of FY'02 Accomplishments:

Review of FY'01 (Bullets):

1. Standardized the **non-quantitative** RT/PCR conditions and demonstrated expression of each of the four neuronal sodium channel genes in normal and injured rat brain.
2. Completed characterization of the conditions for real-time RT/PCR using TaqMan methodology and began our quantitative studies of differential expression of the four sodium channel genes in normal and injured rat brain tissue.
3. Initiated the *in situ* RT/PCR methods.
4. Initiated the *in vitro* antisense oligonucleotides (ASOs) studies.
5. Completed the *in vitro* pharmacological experiments evaluating the neuroprotective properties of the sodium channel blockers mexiletine, QX-314, and the novel blocker RS100642 in three neuronal culture models of neurodegeneration (H/H, veratridine and glutamate). Manuscript was prepared for publication.
6. In parallel experiments, *in vivo* dose-response experiments were completed in the rat MCAo 24 hr recovery model demonstrating the excellent neuroprotection potency and efficacy of systemic post-treatment with RS100642, and of limited efficacy with mexiletine.

Review of FY'02 (Bullets):

1. Completed standardization the TaqMan quantitative RT/PCR (QRT/PCR) conditions during the 1st quarter of FY'02 and subsequently initiated and completed the quantitative characterization of the time-course of expression of each of the four neuronal sodium channel genes in normal and injured rat brain (out to 72 hours post-injury). (Manuscript submitted for Publication)
2. The *in vitro* functional studies using ASOs were begun. Preliminary results indicate that the blocking function expression of either the rBIII, PN1 or PN3 genes using ASOs does not exhibit significant neuroprotection. However, consistent with our in vivo expression results, preliminary results suggest that rBI ASO is neuroprotective. Critically, we have determined that the rBI ASO is also producing increased protease activity and related cytotoxicity in normal cultured neurons, possibly interfering with the neuroprotection. This problem is currently being investigated (see above) and will need to be resolved prior to additional studies being initiated.
3. Initiated comprehensive *in vitro* RT/PCR time-course experiments on the expression of the sodium channel genes.
4. Initiated *in situ* RT/PCR experiments to characterize the regional changes in the localization of the expressed sodium channels in normal and injured rat brain tissue. Although several technical difficulties were encountered (see above) *successful in situ* hybridization studies have now been completed for the rBI sodium channel in the 24 hr injury model (Neuroscience Abstract submitted). Experiments are in progress to study the expression of other sodium channel genes (i.e. rBIII, PN1 and PN3).
5. As a logical extension of our *in vivo* gene expression studies, we have designed and synthesized the primers and probes for other sodium channels of possible functional importance, namely the rBII (rat brain II) sodium channel gene, and the sodium-

calcium exchanger genes NCX1, NCX2 and NCX3. Preliminary studies using TaqMan QRT-PCR assay have been initiated.

6. We have extended *in vivo* MCAo our neuroprotection studies with RS100642 to now include 72 h recovery. In addition, **although not a part of our original research plan** using both pre-treatment and post-treatment protocols we have initiated experiments aimed at addressing the possible benefit of sodium channel blockade to treat the development of post-injury brain seizures and we have discovered potent anti-seizure actions of RS100642.
7. As part of the neuroprotection drug development studies described above in #6, we have also completed EEG neurotoxicity studies of RS100642 in normal, uninjured rats and have determined its safety at doses as high as 600 mg/kg. Again, this was not a part of our original research plan but it was necessary to include these studies as a results of 1) our discovery of brain neurotoxic EEG properties for mexiletine at doses very close to its neuroprotective dose and 2) our recent discovery that non-convulsant brain seizures, similar to what has been reported in human clinical studies, may be a serious consequence of experimental brain injury as well.

3. Details of accomplishments for Year 2:

1. During the past 12 months we completed a full time course study while establishing conditions for successful amplification of four sodium channel genes mRNAs using quantitative RT-PCR using the TaqMan™ technique. The selection of primers, probes, fluorescence dyes and other technical details were listed in the last annual report. The sodium channel genes for expression studies included tetrodotoxin-sensitive (TTX-s) sodium channels, namely, rat brain sodium channel type I and III (rBI and rBIII) and peripheral nerve type 1 and 3 (PN1, PN3). We studied the effect of middle cerebral artery occlusion (MCAo) for two hours followed by reperfusion on expression of these sodium channel genes in various brain regions. The sham controls and injured animals were sacrificed at 2, 6, 24, 48 or 72 hr post-MCAo and their injured and contralateral hemispheres were dissected out.

A. Infarct analysis: In this experimental series all rats survived throughout the experiments. Computer-assisted image analysis was used to digitally image the posterior surface of each TTC-stained forebrain section (Loats Associates, Westminster, MD). Figure 1 shows the representative images of coronal sections demonstrating ipsilateral infarcts in the ischemic hemisphere, and the corresponding non-infarcted contralateral hemisphere, taken from animals at 2 h to 48 h post-MCAo. At 2 h post-MCAo (no reperfusion) there was no obvious ischemic damage to the cerebral tissue. The severity of the ischemic injury was progressive as the reperfusion time extended from 6 h to 48 h post-MCAo.

B. Standard curves: Calibration curves for quantification of sodium channel gene expression were constructed on a 1:2 serial dilution of DNA Template Reagents (Perkin Elmer) (Figure 2; Top). These standard curves demonstrate that quantitation of each target gene was linear on a scale of

at least 7 orders of magnitude with excellent correlation factors of 0.99 and slope values ranged between 3.6-4.2 (Figure 2; Bottom).

C. NaCh gene expression efficiency in normal rat brain: Quantification of the level of relative mRNA for each sodium channel gene was first analyzed in normal rat brain. The mRNA levels of rBI, rBIII, PN1, PN3 and the house-keeping gene β-actin detected by quantitative RT-PCR and shown in Figure 3 demonstrate that rBI is the most abundant of the four sodium channel genes expressed in normal rat brain. Compared to β-actin gene expression, the relative level of expression of each of the NaCh genes were rBI, 18%; rBIII, 8.3%; PN1, 7.3% and PN3, 9.6%. The rBI gene expression was significantly greater than the other NaCh genes, whereas the differences between these channels (i.e. rBIII, PN1 and PN3) were very small and not significant.

D. NaCh gene expression during MCAo injury: NaCh gene expression was quantified at various time points following MCAo and the results are shown in Figure 4 (A-D). There was no significant difference in rBI gene expression in the contralateral (i.e. uninjured) hemisphere (compared to the same hemisphere from sham controls) at any time point studied. However, a significant down-regulation of rBI gene expression was detected in the ischemic hemisphere from 6 h post-MCAo to 48 h post-MCAo with a maximal decrease being observed at 24 h post-injury (Fig 4A).

Compared to sham controls, rBIII and PN1 genes were significantly down regulated in both injured and contralateral hemispheres but only acutely at 2 or 2-6 hr post-MCAo. The PN3 gene was significantly down regulated in only the injured hemisphere at 2-6 h post-MCAo (Fig 4B-4D). However, there was no significant difference in the expression levels of these three NaCh genes at any time in the injured hemisphere when compared with their contralateral hemispheres.

2. Initiated the *in vitro* functional studies using ASOs for each sodium channel gene in neuronal culture models of sodium activation neurotoxicity. For these experiments, during FY'01 we developed and synthesized (commercially) the ASO for each of the four sodium channel genes. Studies using these ASOs were initiated using primary cultures of forebrain neurons (after 7 days in culture) and hypoxia/hypoglycemia (H/H) and veratridine models of excitotoxicity/injury. Increasing doses (0.05-5.0 μM) of these ASOs were used to pre-treat cells (4-24 hr) prior to injury. No significant neuroprotection was observed using rBIII, PN1 or PN3 ASO (at any concentrations or pre-treatment time intervals studied) in either injury model. This appears consistent with our *in vivo* experiments where little significant change in the expression these genes was seen following MCAo brain injury. On the other hand, the rBI ASO provided a limited neuroprotection when applied to neurons at 1-5 μM concentrations 24 hr before the insult/injury; this result is consistent with our observed changes in the rBI gene expression profile following brain injury. Studies are currently in progress to determine the neuroprotective effects of longer pretreatment periods (36 or 48 hr) using lower ASO

concentrations (0.1-1 μM). Once the optimal treatment period is established, additional studies will be undertaken to determine if a combination of rBI ASO with other sodium channel gene ASOs might improve the degree of neuroprotective. However, as mentioned above the rBI ASO treatment also produced a mild degree of cytotoxicity associated with increased protease activity that resulted in cell detachment from the culture wells. This situation was unique for only the rBI ASO, as the other three sodium channel gene ASOs did not produce any toxicity and had no effect on protease activity. This problem likely increased the variability of our results. The planned studies using lower ASO concentrations and longer pre-treatment intervals will hopefully overcome this problem.

3. Initiated comprehensive *in vitro* RT/PCR time-course experiments on the expression of sodium channel genes in cultured neurons. We have determined that the expression profile of these four sodium channel genes is quite different in cultured neurons as compared to that in adult rat brain tissue. We earlier reported the expression profile of these sodium channel genes in adult rat brain with rBI exhibiting highest expression followed by rBIII>PN1>PN3. However, in primary cultures of forebrain neurons PN3 exhibits the highest expression level followed by rBIII and rBI, and the lowest expression is for PN1 gene. In several separate experiments, cells were treated either with vehicle (Locke's solution) or 20, 10 or 5 μM veratridine (providing approximately 85%, 60% and 48% cell death) for 45 min and cells were harvested for expression studies at 4, 12 or 24 hr after the insult. In preliminary studies, 4 or 12 hr post-treatment did not exhibit any significant changes in expression of any of the NaCh genes (Data not shown). Furthermore, at 20 μM concentration of veratridine there was >80% cell death and the amount of RNA recovered for RT/PCR studies was very small and provided variable results (Data not shown). Hence, for subsequent studies 10 or 5 μM veratridine and 24 hr post-treatment period were used. In preliminary studies, treatment of neurons with 10 μM veratridine produced down-regulation of rBI and PN3 gene expression 24 hr post-insult (**Figure 5**). The down-regulation of rBI gene in cultured neurons following veratridine insult is consistent with our *in vivo* findings reported above. In the above study, neurons were exposed to veratridine for 45 min only. Studies are now currently under progress to determine the effect of longer exposure periods (24 hr) to veratridine on expression of these genes.

4. Initiated *in situ* hybridization experiments to visualize the changes in sodium channel gene expression following MCAo injury at cellular and brain region levels. After extensive adjustments and modifications to our experimental protocol, we established successful methods for our *in situ* studies. Of critical importance we determined that prior staining with TTC was not optimal for conducting subsequent *in situ* preparation and analysis therefore new brain tissue is being generated for these experiments. For these studies animals are perfused with 4% paraformaldehyde after an intraperitoneal injection of ketamine (100 mg/kg). The brains are removed and stored in 20% sucrose/3% paraformaldehyde at 4°C overnight. Tissue blocks from brain can then be embedded in paraffin and cut at 10-μm thickness. The *in situ* hybridization technique used has been reported earlier (Rollwagen et al, 1998). Basically, sections are de-paraffiized and permeabilized with proteinase K (2.5 μg/ml) for 10 minutes at 37°C

followed by acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 minutes at room temperature. Hybridization is performed overnight at 37°C with a sodium channel gene-biotinylated DNA probe. Post-hybridization washes (1X Stringent Wash Solution) are undertaken for 30 min at 45°C. After blocking nonspecific biding sites with blocking butter [3% BSA, 0.3% Tween 20, in 50 mM Tris buffer (pH 7.4)/200 mM NaCl)], the signals are detected immunochemically by subsequent incubation with streptoavidin-alkaline phosphatase (AP) conjugate (DAKO Corp.). The purple blue color reaction was developed in bromochloroindolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) substrate.

In this study, the sodium channel oligonucleotide probes (synthesized by Genset Oligos Inc, La Jolla, CA) were complementary to rBI mRNA nucleotide 1113-1146, rBIII 2118-2143, PN1 1472-1498, PN3 1565-1588 and β-actin 37-64. A Blast search of the EMBL/Genbank databases indicated that there was no close homology between the probes directed against the sodium channel genes respectively and β-actin gene, or indeed any other sequences registered within these databases. The probes were 5' end-labeled with biotin. The biotinylated poly T Probe (Research Genetics, Huntsville, AL) was used as a positive control, and negative controls consisted of using a sense probe.

The results of this study are presented in **Figures 6-13**. Figure 6 shows a representative section of injured rat brain (24 hr post-MCAo) stained with H/E staining (1.25x). In this section the injured area is stained light pink and the uninjured (contralateral) areas are stained bright pink. The pattern of staining is quite similar to what we routinely observe with TTC staining. Figure 7 shows another section of injured rat brain stained with crystal violet (CV) staining. The pattern of staining is again very similar to that seen with both H/E and TTC staining. Figure 8 is a representative *in situ* hybridization positive control of the injured rat brain, and Figure 9 is the *in situ* hybridization negative control. Figure 10 is a representative β-actin *in situ* hybridization of a sham control rat brain (areas of dark blue staining shows the presence of β-actin mRNA; 1.25x). Figure 11 shows a representative *in situ* hybridization of the rBI gene in an injured rat brain section at low power (1.25x). This result shows that the rBI gene is not only down-regulated in the brain areas within the core injury site, but also in proximal sites. Critically, our in situ experiments have shown for the first time that there also a dramatic down-regulated (approaching 20-30% of normal) of the rBI channel in the contralateral hemisphere following injury. This is an extremely exciting and novel finding suggesting that what is normally (based on classical histopathologically) considered as uninjured (i.e. countralateral) brain tissue is indeed exhibit significant damage at the molecular "ion channel" level. Figures 12 and 13 describe the rBI *in situ* hybridization of subcortical regions of injured and contralateral hemispheres at higher power, showing clear down-regulation of this NaCh gene following MCAo injury (4 x). Additional *in situ* experiments evaluating the other NaCh genes (namely, rBIII, PN1 and PN3) following MCAo injury are in progress.

5. Designed and synthesized probes and primers for more genes. As mentioned earlier, as a logical extension of our current studies, we have planned to study the expression of other critical ion channel regulation related genes. We have already designed and

synthesized (commercially) sets of primers and probes for QRT/PCR studies of rBII gene and three sodium calcium exchanger genes (NCX1, NCX2 and NCX3). **Figure 14** shows the sequences of these primers and probes. Initial studies using these primers and probes will start soon.

6. Using the 72 h brain injury and recovery model, we demonstrated that an optimally neuroprotective dose of RS100642 (determined from the 24 h D-R studies completed during FY'01) was effective in reducing infarction (Figure 15) and improving functional recovery (Figure 17), and attenuated the post-injury development of brain seizures.

1. Both pre- and post-MCAo treatment with RS100642 (1.0 mg/kg) resulted in a significant decrease in cerebral infarction when evaluated after 72 h of MCAo/reperfusion (Figure 16). The reduction of total and core infarct volumes corresponded to 51 ± 14% and 60 ± 18% (pre-treatment group) and 46 ± 12% and 65 ± 14% (post-treatment group), respectively. Similarly, the overall percent hemispheric infarction was significantly reduced (total = 16%, core = 6%, pre-treatment) and (total = 17%, core = 5%, post-treatment). No significant effect on cerebral edema was measured as compared to the vehicle group. For the pre- and post-treated groups there was a significant improvement in neurological recovery at 72 h post-MCAo, NS = 0.6 ± 0.2 and 0.5 ± 0.3, respectively (Figure 17) where two animals in each group exhibited complete recovery of neurological function (NS=0). Compared to mexiletine, RS100642 was approximately 10x more potent at equally effective doses (Figure 18).

2. Using our EEG analysis procedures (see Figure 19 for high resolution electrode placement) we demonstrated that MCAo-injured animals exhibited non-convulsant seizures (NCS), predominately during the first hour of injury, which were attenuated following reperfusion of blood to the brain (as observed from EEG recordings). It is critical to emphasis that these injured animals do not elicit behavioral convulsant activity during the observed brain seizures (hence, the need to monitor spontaneous cortical function via EEG methods), and in general animals are conscious and ambulatory although occasionally wet dog shake (WDS) behavior was observed during NCS. The NCS were quantified as generalized spike/slow-wave complexes occurring at a frequency of 1-2 per second and lasting 134 ± 33 s with 4.2 ± 1.2 ictal events occurring in 4 out of 5 vehicle-treated animals. On average, NCS began 26 ± 6 min following occlusion of the MCA in vehicle-treated animals. Furthermore, NCS were generalized to the entire brain and were recorded from all 10 cortical electrodes. Each consecutive seizure spike gradually increased in amplitude and following resolution of seizure activity a period of EEG post-ictal depression occurred. **Pre-treatment with RS100642 (1.0 mg/kg) completely blocked all NCS activity. With 30 min post-MCAo treatment of RS1000642 (1.0mg/kg), 3 of 4 animals experienced NCS but only 1 event occurred per animal and was shorter in duration (39 ± 12 s, $p<0.05$, independent t-test as compared to vehicle group).** These results are summarized in Figures 20 and 21.

KEY RESEARCH ACCOMPLISHMENTS for FY'02

- Manuscript entitled "RS-100642-198, a novel sodium channel blocker, provides differential neuroprotection against hypoxia/hypoglycemia, veratridine or glutamate mediated neurotoxicity in primary cultures of rat cerebellar neurons" was accepted and published in Neurotoxicity Research **(In Press)** 2001.

- Manuscript entitled "Differential pattern of expression of voltage-gated sodium channel genes following ischemic brain injury in rats" **was written and submitted for publication.**

- Manuscript entitled "Neuroprotective effects of the sodium channel blocker RS100642 and attenuation of ischemia-induced brain seizures in the rat" **was written and submitted for Institute Clearance.**

- Abstracts entitled "Expression of sodium channel genes following ischemic injury: an *in situ* hybridization study" and "EEG characterization of non-convulsive seizures following middle cerebral artery occlusion in the rat" **were submitted for presentation to the 31st Annual meeting of the Society for Neuroscience.**

- Completed the quantitative characterization of the time-course of expression of each of the four neuronal sodium channel genes in normal and injured rat brain.

- Initiated the *in vitro* RT/PCR gene expression experiment and the functional *in vitro* Antisense Oligonucleotide experiments for each of the four sodium channels.

- Established the methodology procedures for the *in situ* hybridization experiments and completed the rBI studies. Initiated the *in situ* hybridization experiments for rBIII and PN1 and PN3.

- Completed *in vivo* neuroprotection experiments for RS100642 in the 72 recovery model, initiated and completed EEG evaluation of brain injury induced seizures, and initiated and completed brain injury/EEG seizure experiments defining anti-seizure properties of sodium channel blockade using RS100642.

REPORTABLE OUTCOMES for FY'02

One manuscript published (see appendices)
Two manuscripts submitted (see appendices)
One Abstract submitted (see appendices)

CONCLUSIONS: **SEE ABOVE**

REFERENCES: **UNCHANGED FROM ORIGINAL PROPOSAL**

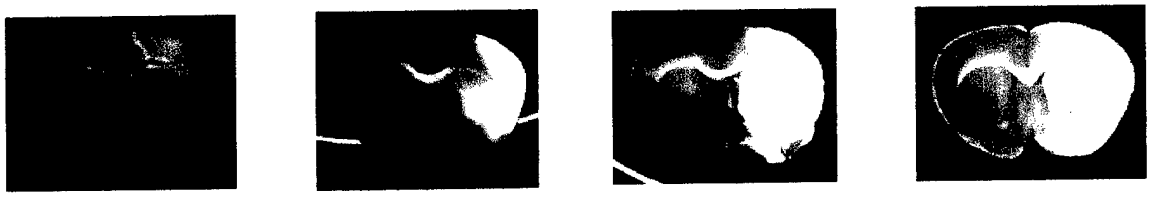
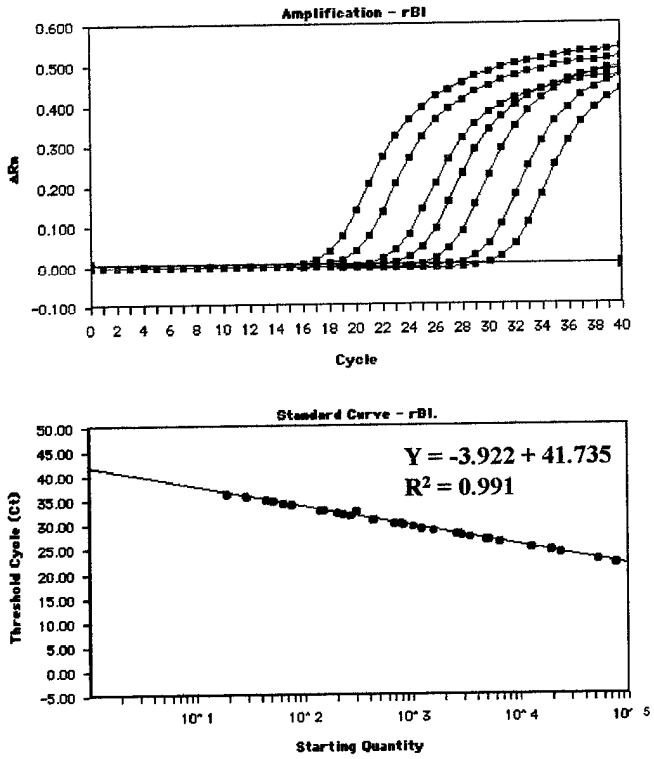


Figure 1


Amplification - rBI
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0.500
0.400
0.300
0.200
0.100
0.000
-0.100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle
Standard Curve - rBI.
Y = -3.922 + 41.735
R² = 0.991
Threshold Cycle (Ct)
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
10^1
10^2
10^3
10^4
10^5
Starting Quantity

Figure 2

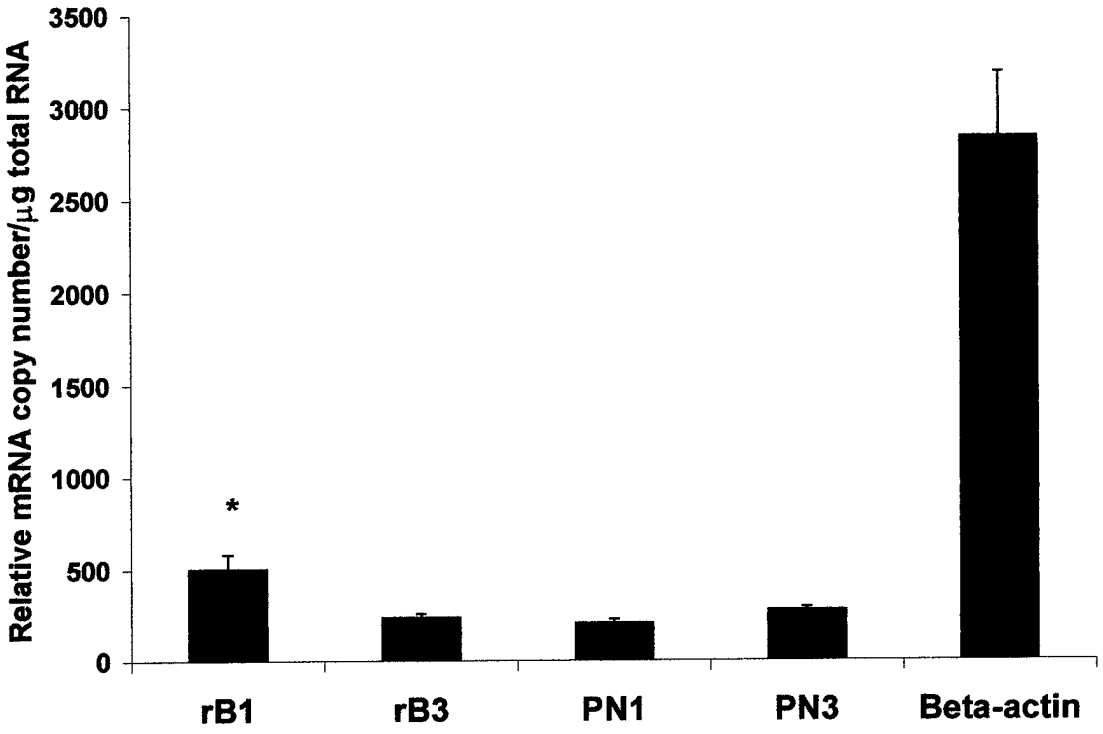

Relative mRNA copy number/μg total RNA
3500
3000
2500
2000
1500
1000
500
0
*
rB1
rB3
PN1
PN3
Beta-actin

Figure 3

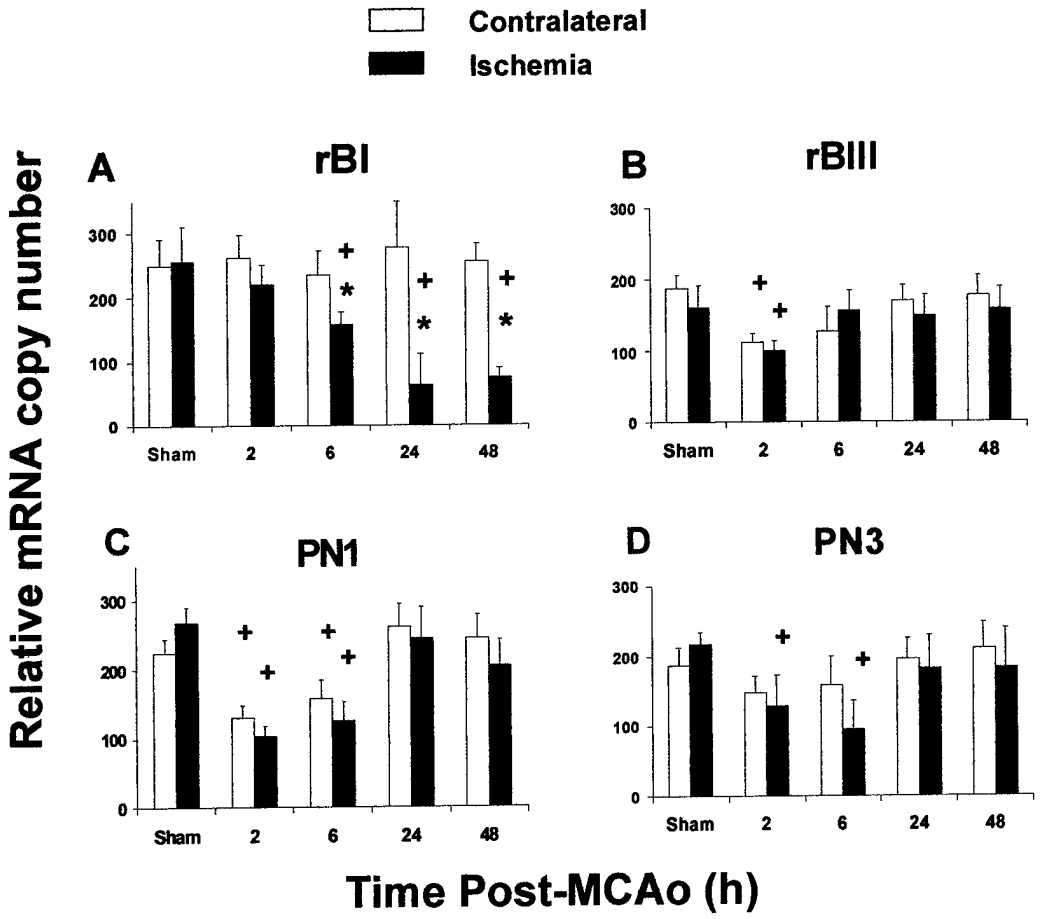
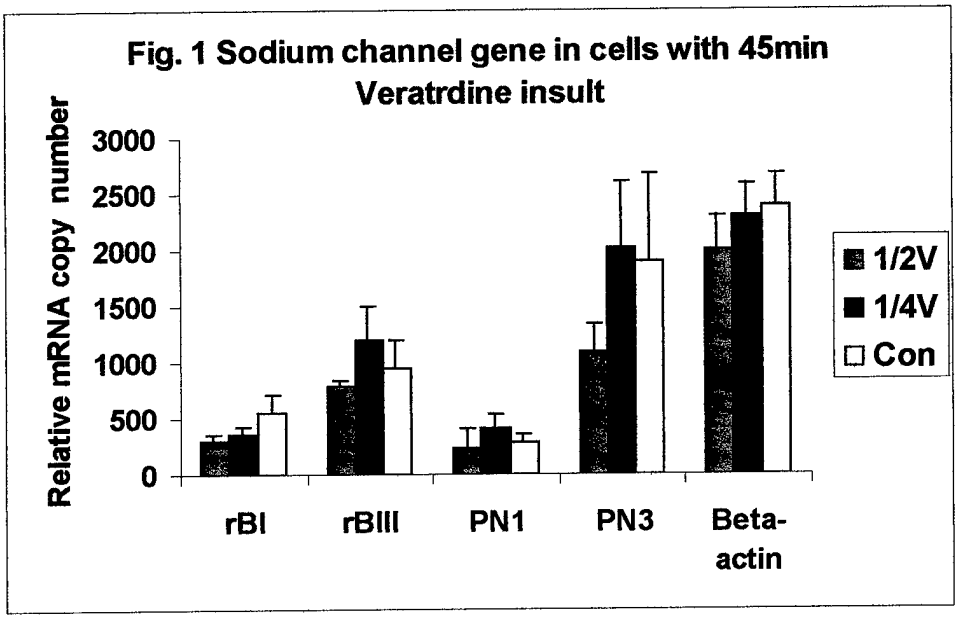

Contralateral
Ischemia
A
rBI
B
rBIII
C
PN1
D
PN3
Relative mRNA copy number
Time Post-MCAo (h)
Sham
2
6
24
48
0
100
200
300

Figure 4



½ V = 10 μM Veratridine
¼ V = 5 μM Veratridine

Figure 5

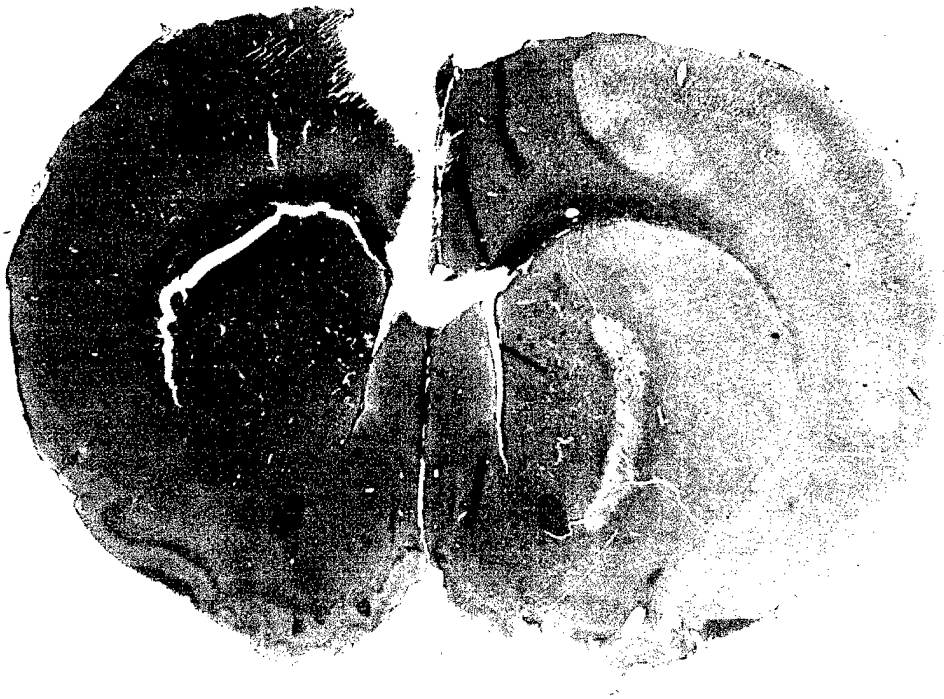


Figure 6

Figure 7

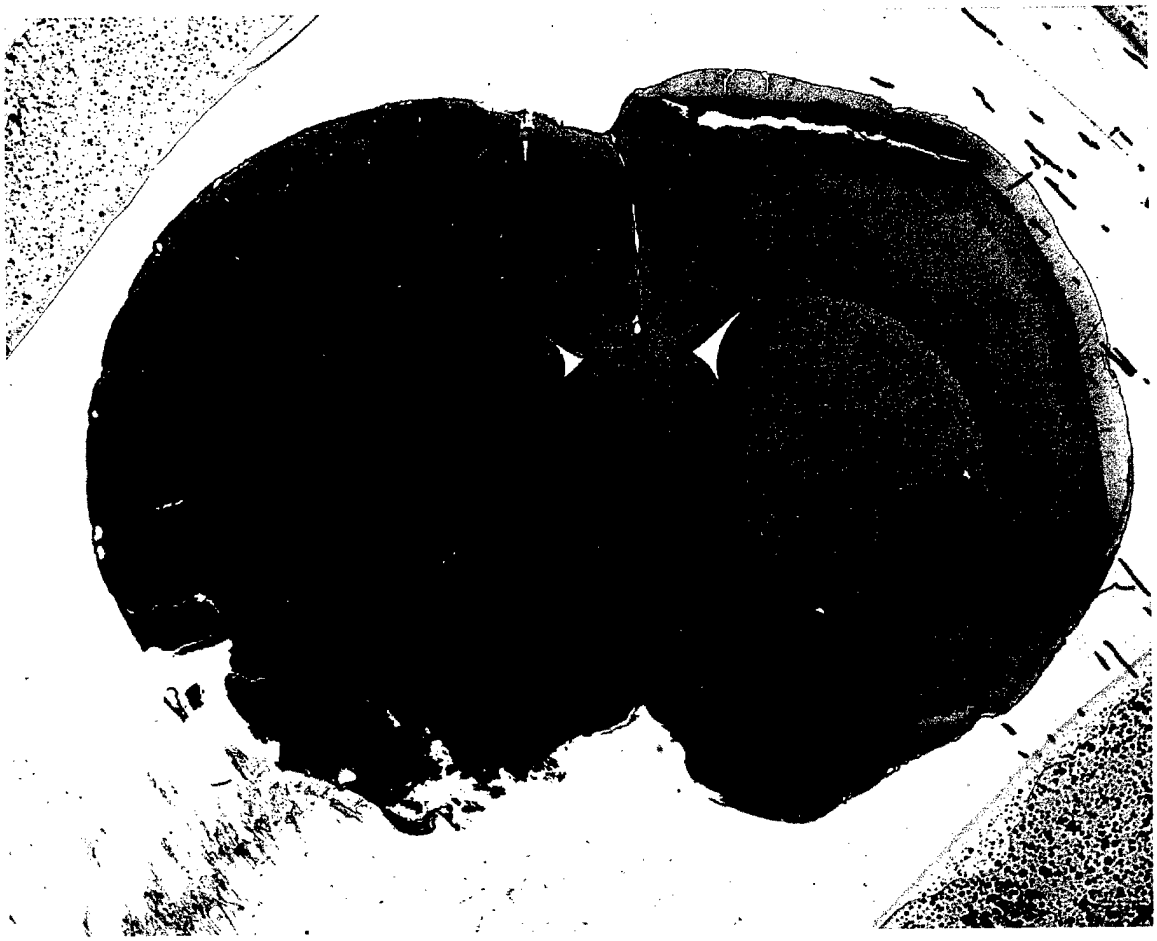


Figure 8

Figure 9

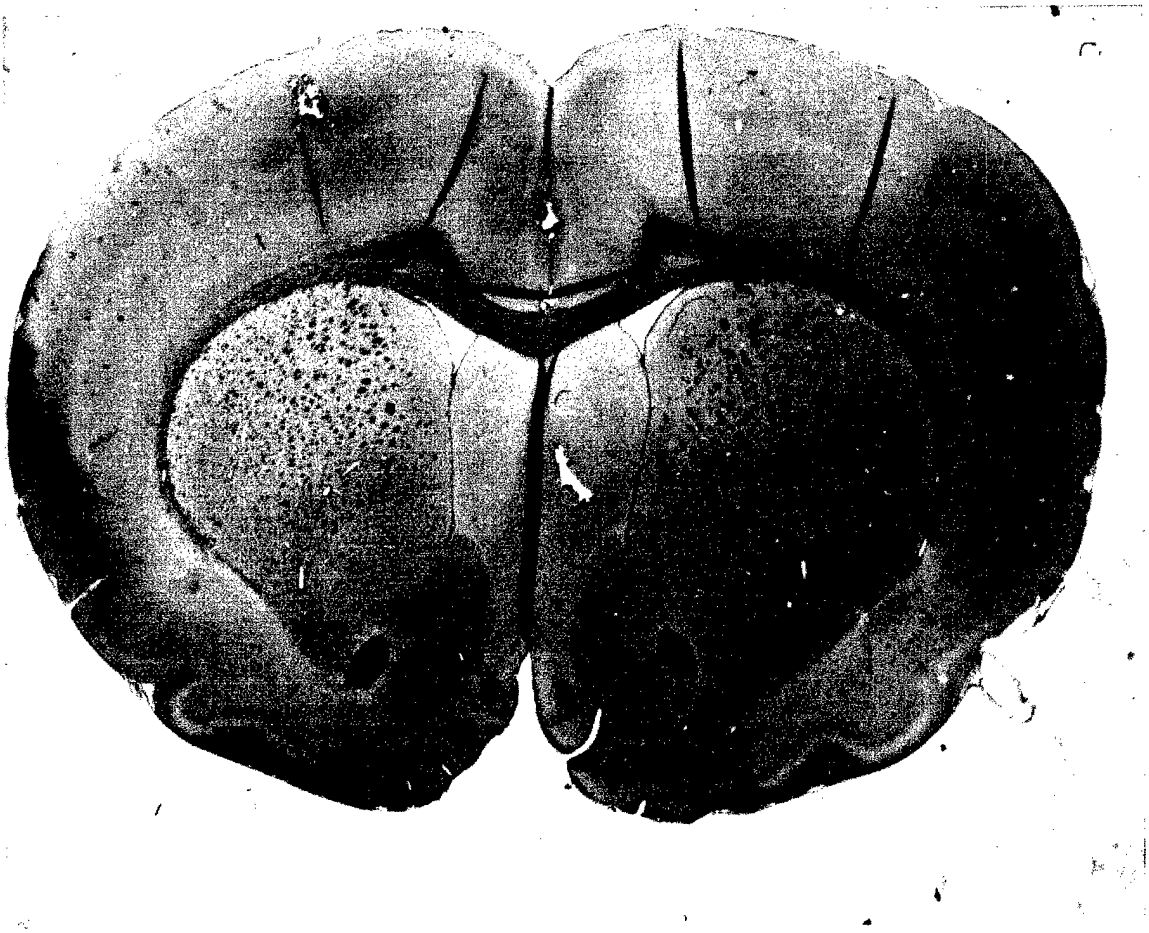


Figure 10

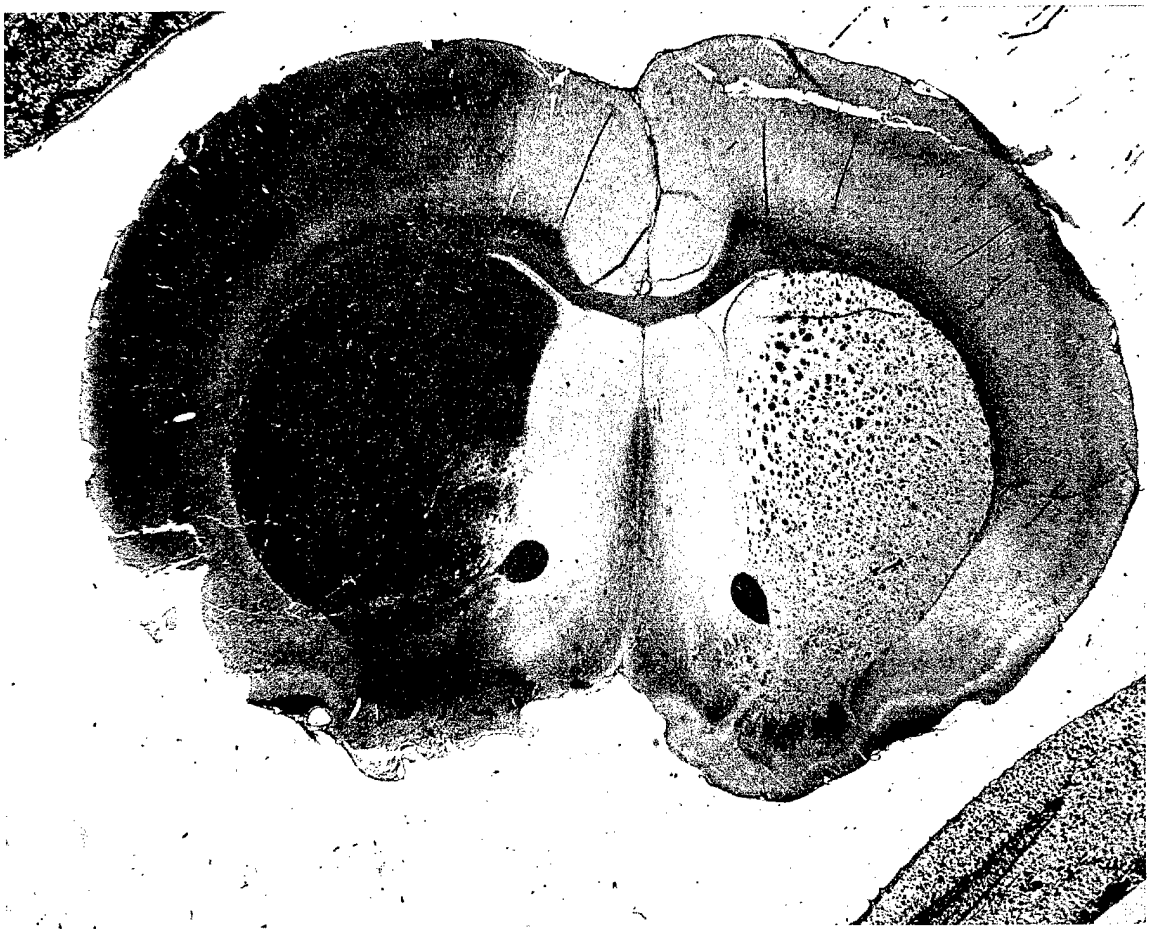


Figure 11

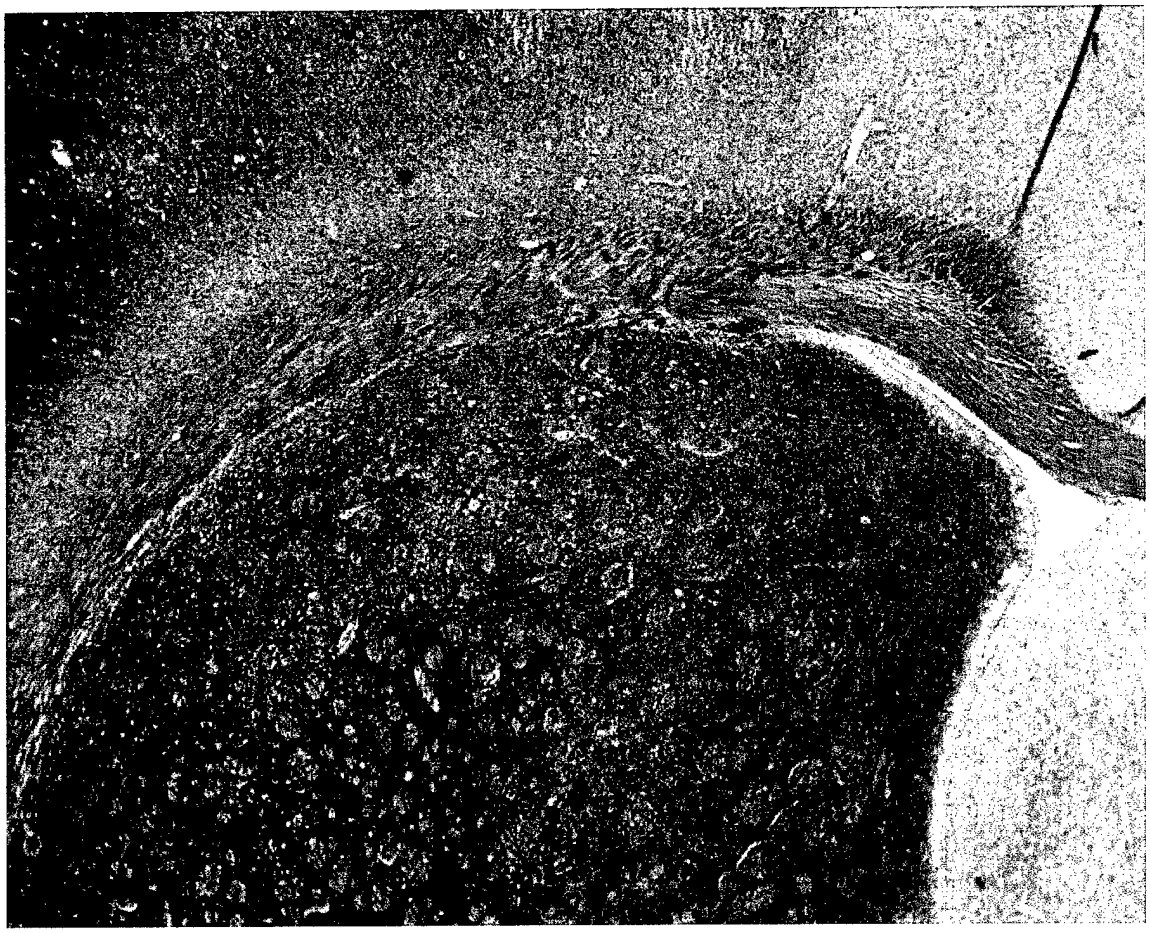


Figure 12

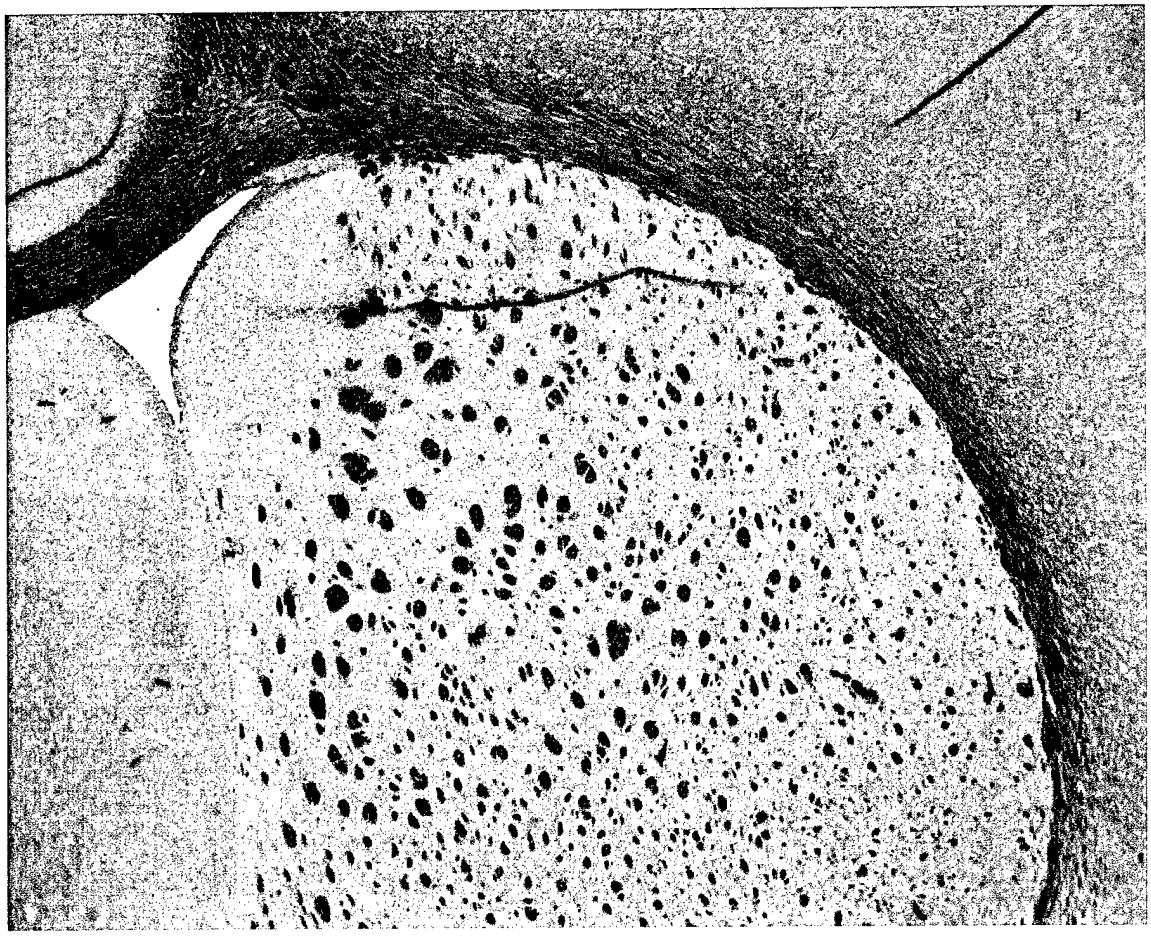


Figure 13

Gene	Primer/probe	Sequence (5'-3')	Base pairs
rBII	forward primer	GAA GCT GTC ACC GCT GCA T	19
	reverse primer	TCC CTG TTA GAC TTC TGG ACA GCA	24
	probe	CTC TCT TGC TGA TGC CGC TGT ATC TTA ACC	30
NCX1	forward primer	GTC TGC GAT TGC TTG TCT CG	20
	reverse primer	GCC CGC GTG GTC ATC A	16
	probe	ACC CAA CAC TGC CAC CAT AAC CAT TTT TG	29
NCX2	forward primer	CTT TGG TGT CTG CAT CCT GGT	21
	reverse primer	CAC CCA AAG TGT GAG GCC A	19
	probe	TCT GCT CAC TGC CCT CAT CGG AGA	24
NCX3	forward primer	GGG TAT TGG TTT GGC CTG G	19
	reverse primer	CAT GGA ACT CCT GTC CCT GC	20
	probe	CCG TCG CCG CCA TCT ACT GGG	21

Figure 14

Figure 15

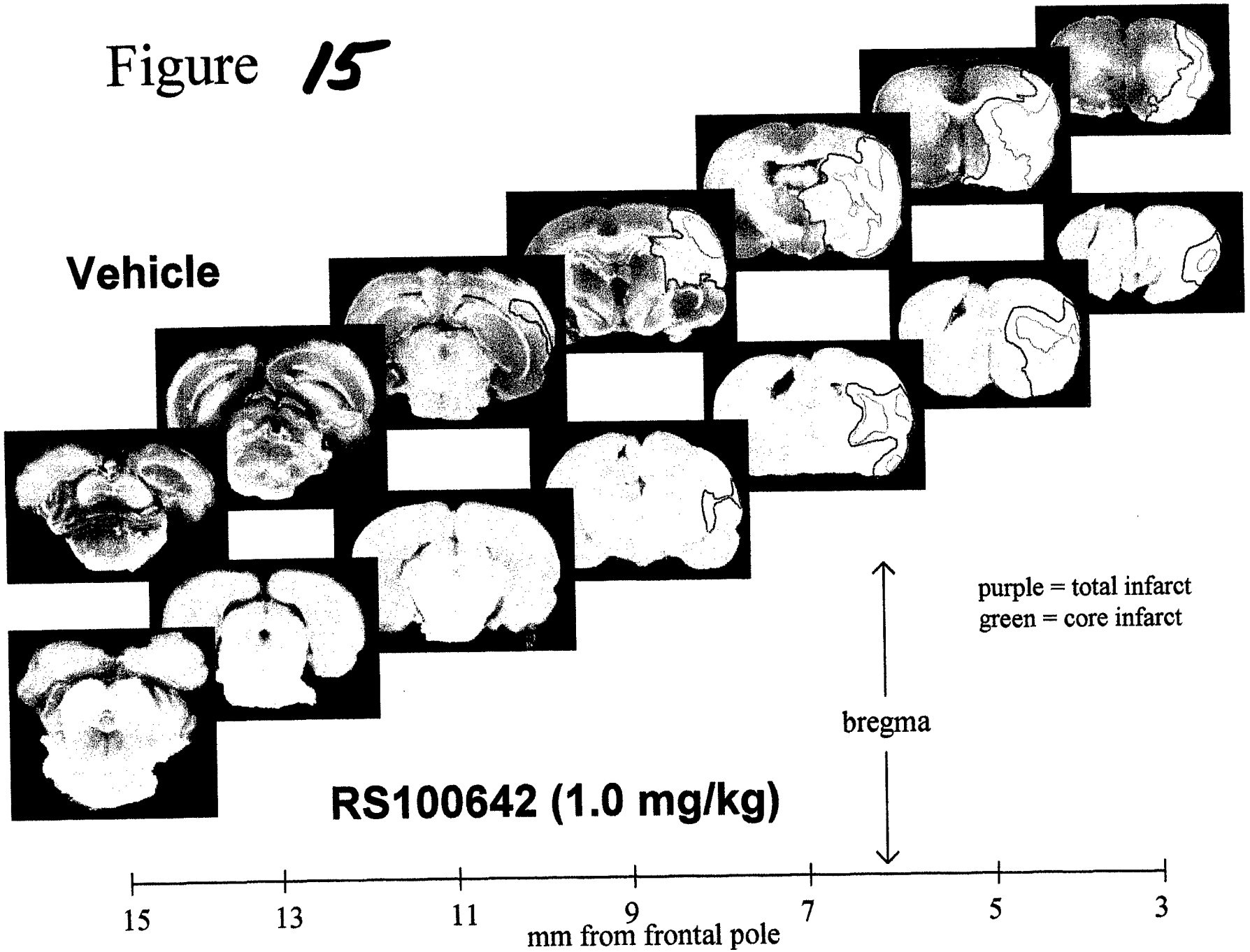

Vehicle
RS100642 (1.0 mg/kg)
purple = total infarct
green = core infarct
bregma
15
13
11
9
7
5
3
mm from frontal pole

Figure 16

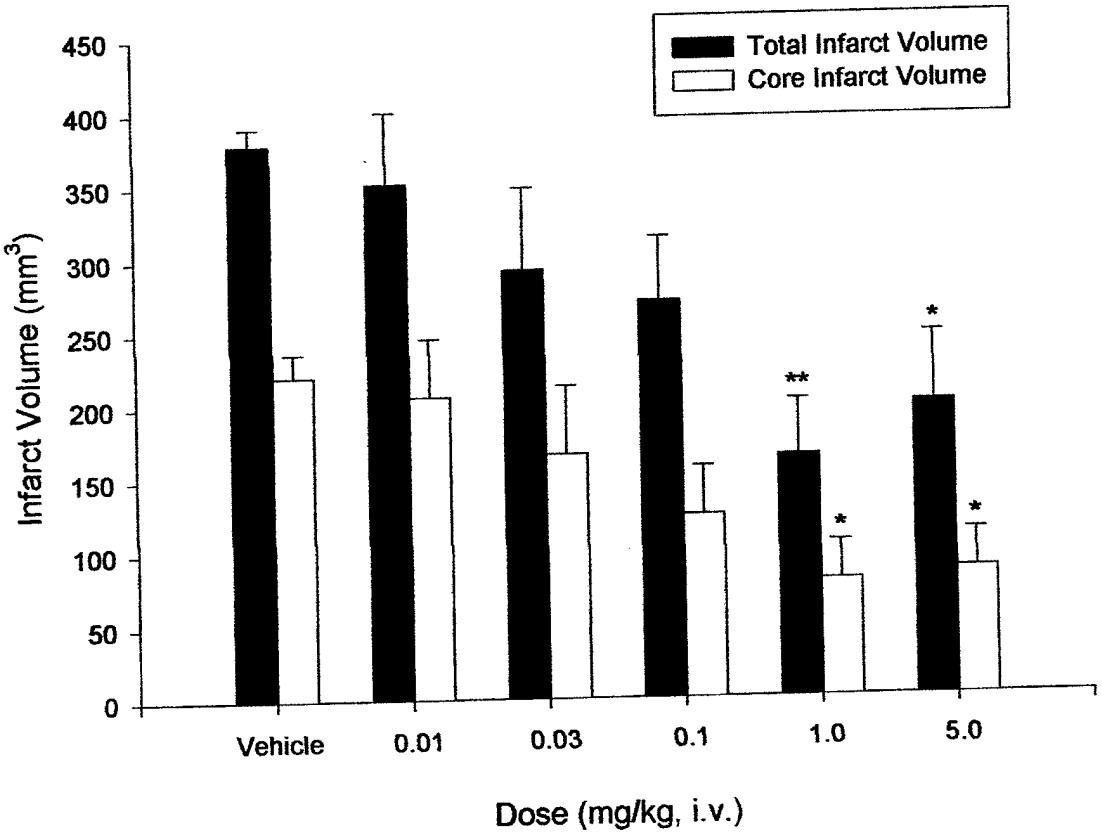

Total Infarct Volume
Core Infarct Volume
Infarct Volume (mm3)
450
400
350
300
250
200
150
100
50
0
**
*
*
*
Vehicle
0.01
0.03
0.1
1.0
5.0
Dose (mg/kg, i.v.)

Figure 17

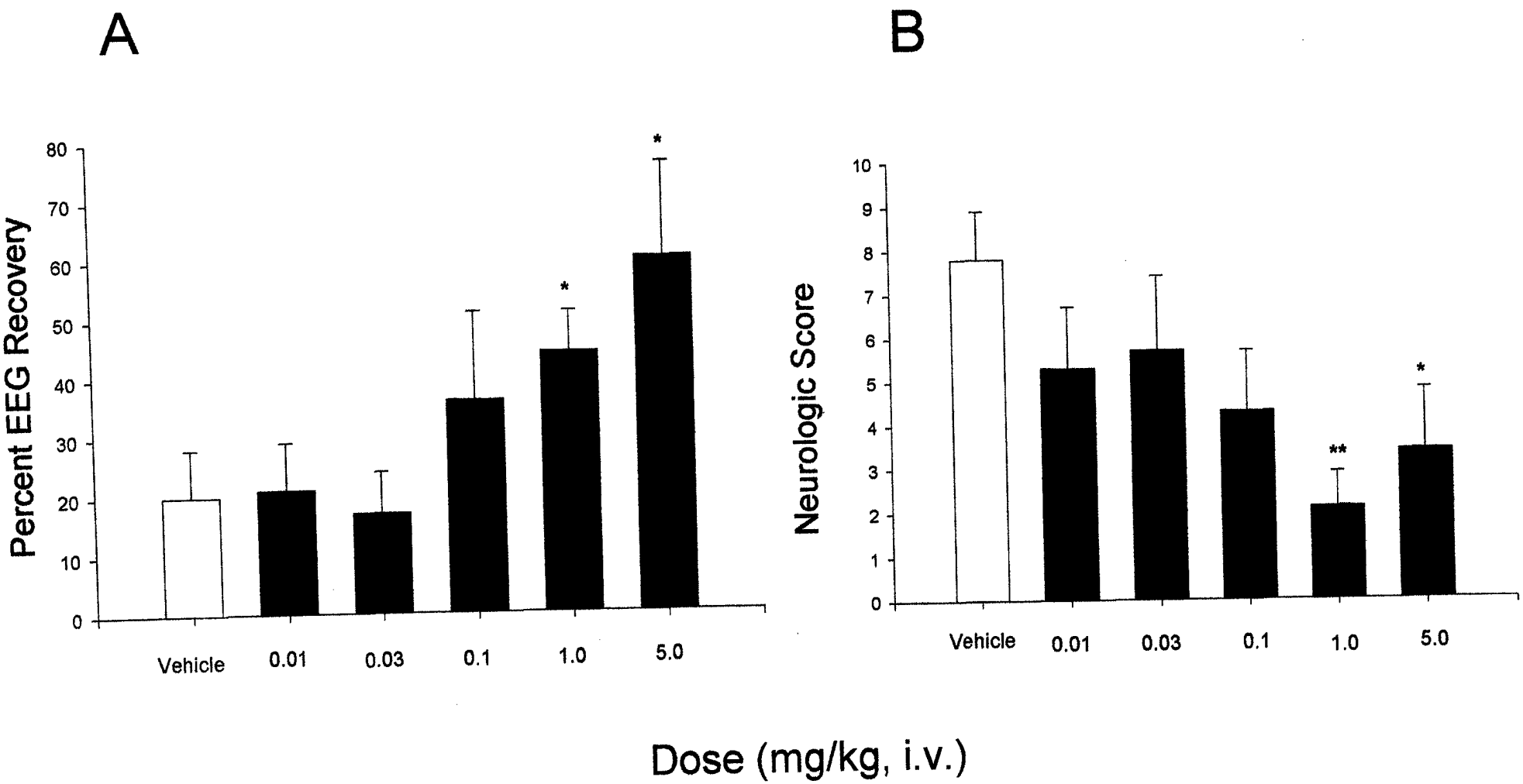

A
B
Percent EEG Recovery
Neurologic Score
0
10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
8
9
10
*
*
*
**
Vehicle
0.01
0.03
0.1
1.0
5.0
Dose (mg/kg, i.v.)

Figure 18

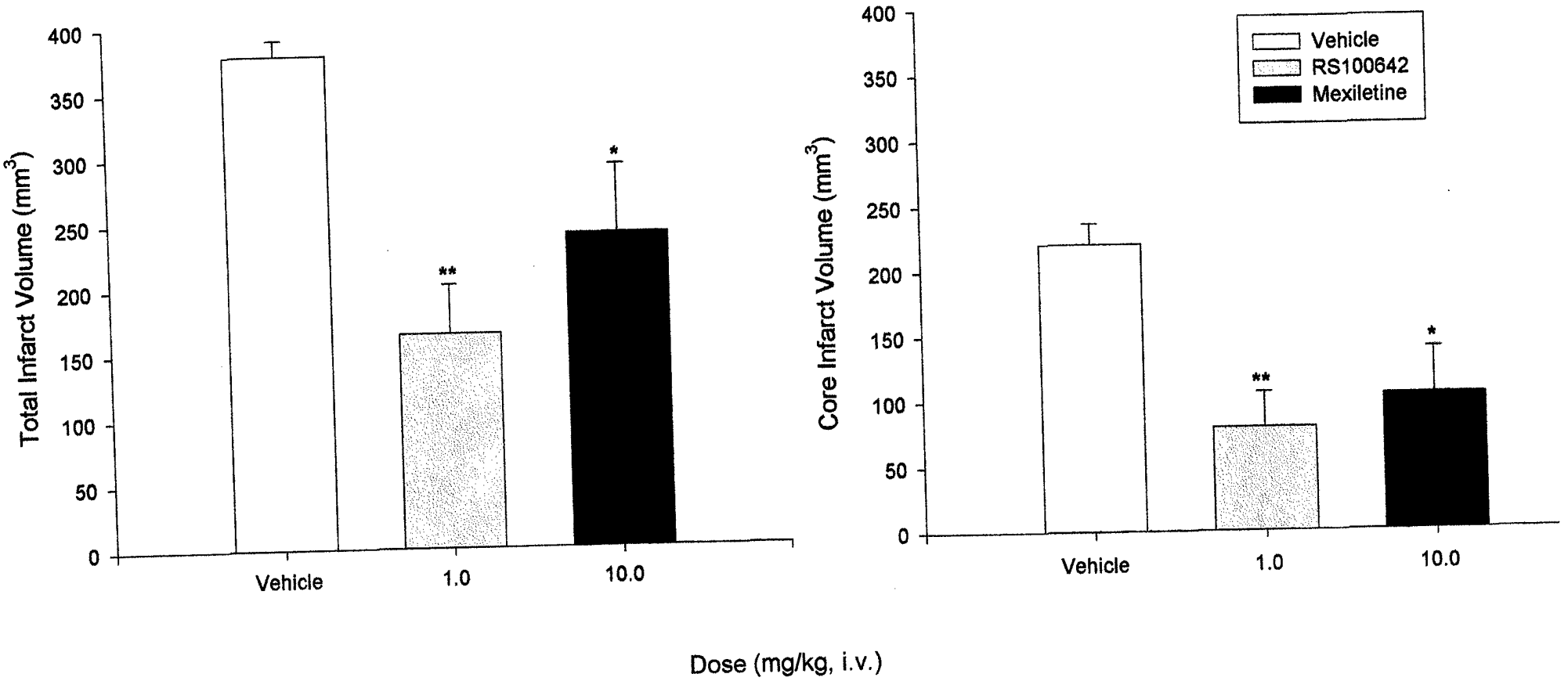

Total Infarct Volume (mm3)
400
350
300
250
200
150
100
50
0
**
*
Vehicle
1.0
10.0
Core Infarct Volume (mm3)
Vehicle
RS100642
Mexiletine
**
*
Dose (mg/kg, i.v.)

Figure 19

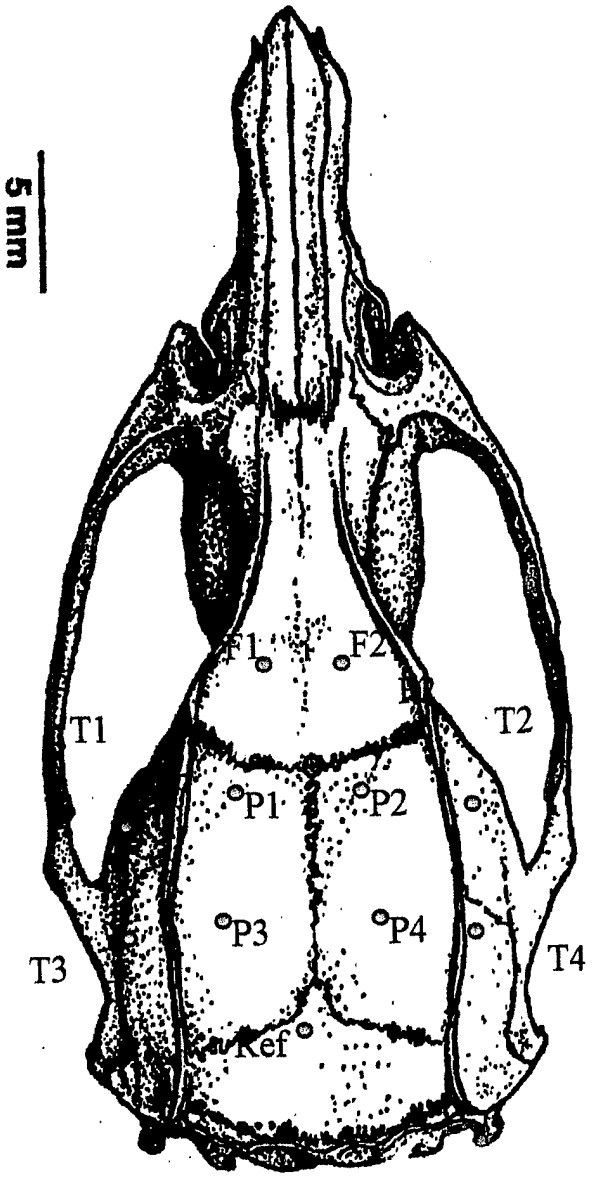


Figure 20

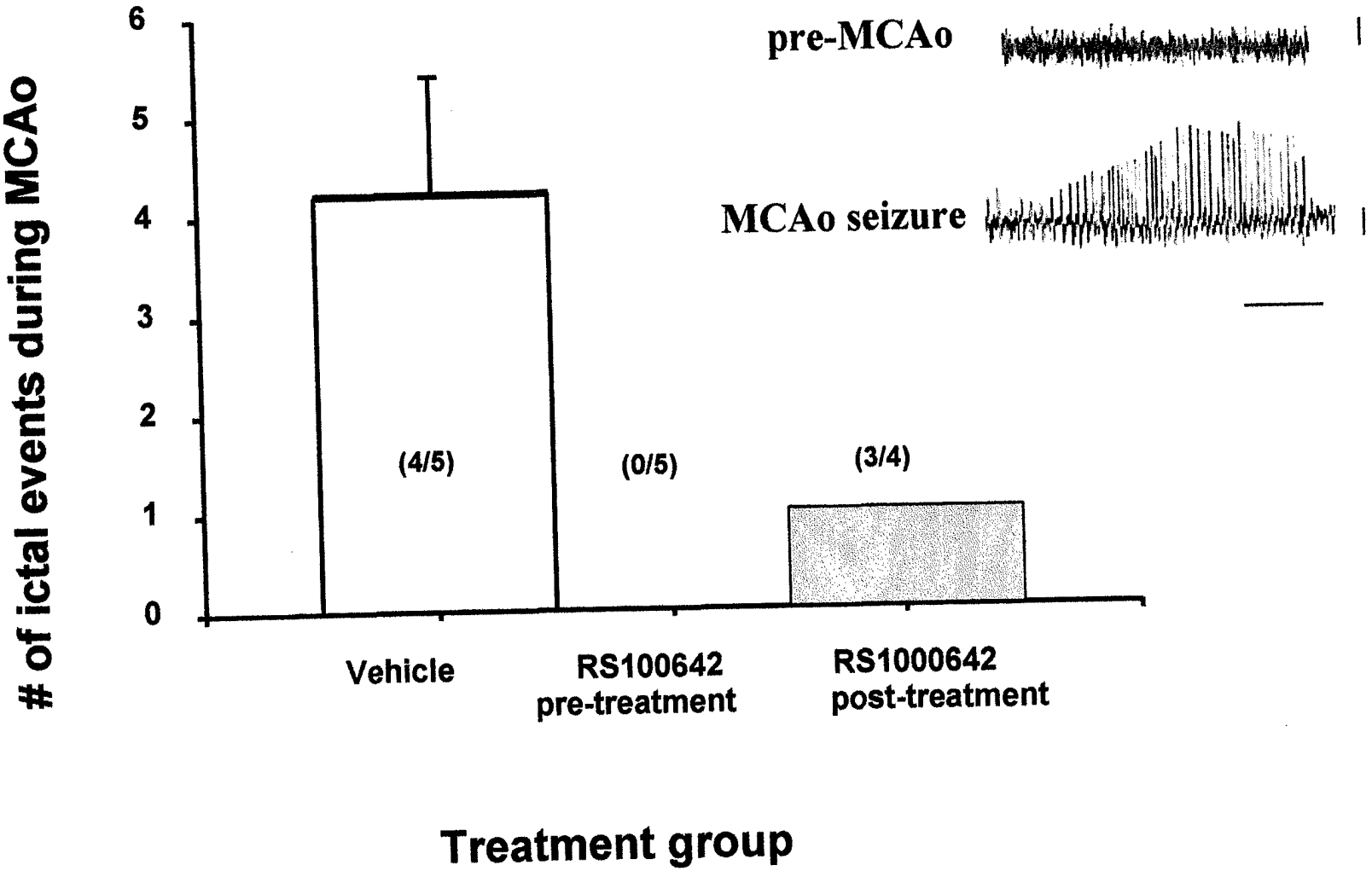
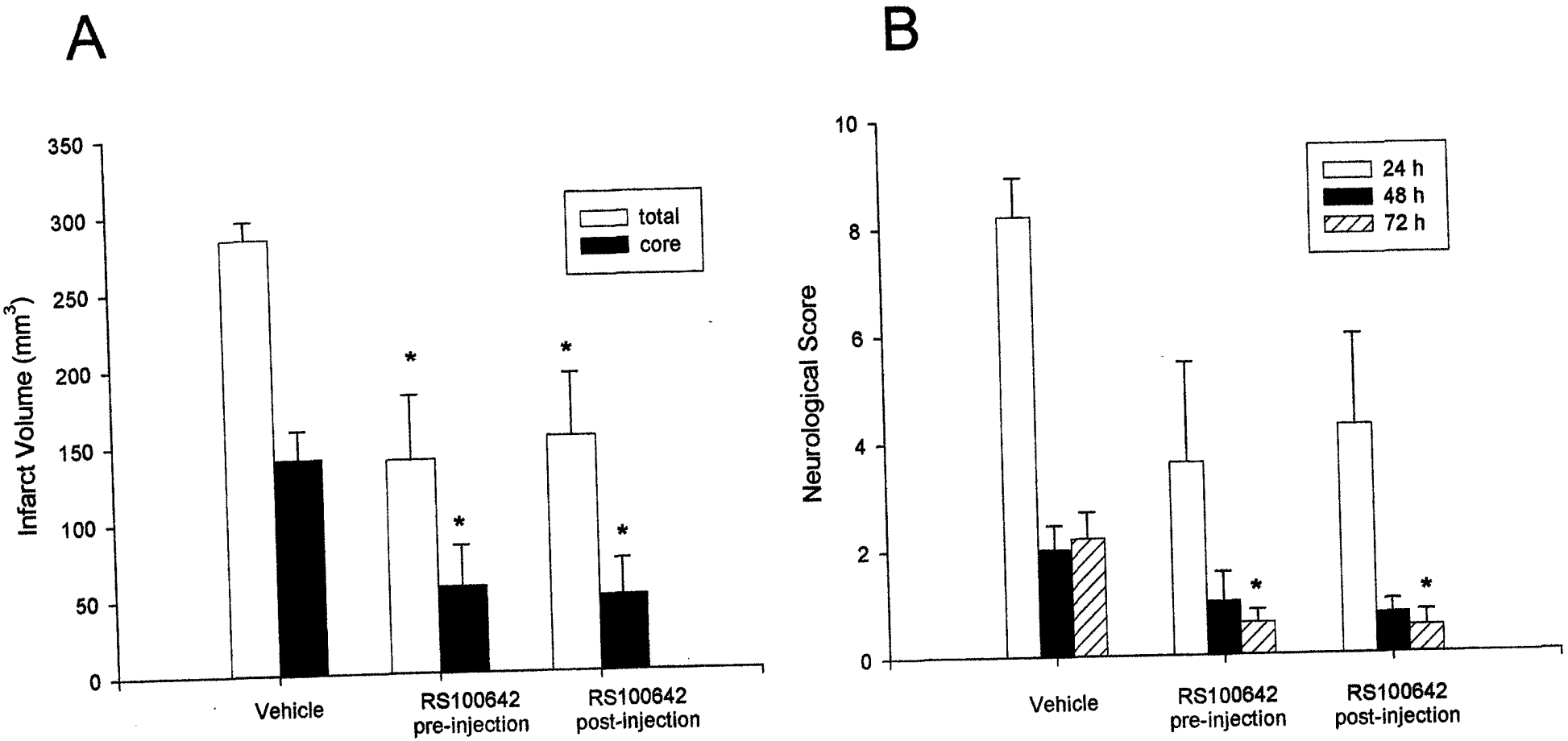

pre-MCAo
MCAo seizure
# of ictal events during MCAo
6
5
4
3
2
1
0
(4/5)
(0/5)
(3/4)
Vehicle
RS100642
pre-treatment
RS1000642
post-treatment
Treatment group

Figure 21


A
B
Infarct Volume (mm3)
350
300
250
200
150
100
50
0
total
core
*
Vehicle
RS100642
pre-injection
RS100642
post-injection
Neurological Score
10
8
6
4
2
0
24 h
48 h
72 h
Vehicle
RS100642
pre-injection
RS100642
post-injection

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RS-100642–198, a Novel Sodium Channel Blocker, Provides Differential Neuroprotection Against Hypoxia/Hypoglycemia, Veratridine or Glutamate-Mediated Neurotoxicity in Primary Cultures of Rat Cerebellar Neurons*

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The present study investigated the effects of RS-100642–198 (a novel sodium channel blocker), and two related compounds (mexiletine and QX-314), in *in vitro* models of neurotoxicity. Neurotoxicity was produced in primary cerebellar cultures using hypoxia/hypoglycemia (H/H), veratridine or glutamate where, in vehicle-treated neurons, 65%, 60% and 75% neuronal injury was measured, respectively. Dose-response neuroprotection experiments were carried out using concentrations ranging from 0.1–500 μM. All the sodium channel blockers were neuroprotective against H/H-induced injury, with each exhibiting similar potency and efficacy. However, against veratridine-induced neuronal injury only RS-100642–198 and mexiletine were 100% protective, whereas QX-314 neuroprotection was limited (i.e. only 54%). In contrast, RS 100642–198 and mexiletine had no effect against glutamate-induced injury, whereas QX-314 produced a consistent, but very limited (i.e. 25%), neuroprotection. Measurements of intraneuronal calcium ($[Ca^{2+}]_i$) mobilization revealed that glutamate caused immediate and sustained increases in $[Ca^{2+}]_i$ which were not affected by RS-100642–198 or mexiletine. However, both drugs decreased the initial amplitude and attenuated the sustained rise in $[Ca^{2+}]_i$ ~~mobilization~~ produced by veratridine or KCl depolarization. QX-314 produced similar effects on glutamate-, veratridine- or KCl-induced $[Ca^{2+}]_i$ dynamics, effectively decreasing the amplitude and delaying the initial spike in $[Ca^{2+}]_i$, and attenuating the sustained increase in $[Ca^{2+}]_i$ mobilization. By using different *in vitro* models of excitotoxicity, a heterogeneous profile of neuroprotective effects resulting from sodium channel blockade has been described for RS-100642–198 and related drugs, suggesting that selective blockade of neuronal sodium channels in pathological conditions may provide therapeutic neuroprotection against depolarization/excitotoxicity via inhibition of voltage-dependent Na^+ channels.

Keywords: Excitotoxicity; Ischemia; Neuronal cultures; Neuroprotection; Neurotoxicity; Sodium channel blockers

* Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, NIH publication 85–23. the views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4–3), AR 360–5.

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INTRODUCTION

It is widely accepted that neuronal cell death results from a cascade of events, ranging from excessive presynaptic release of excitatory amino acids (EAA), alterations in cellular ionic dynamics, toxic postsynaptic accumulation of intracellular calcium $[Ca^{2+}]_i$, and ultimately the activation of secondary signaling mechanisms leading to acute or delayed injury processes (Choi, 1987; DeCoster *et al.*, 1992; Mattson *et al*, 1992). This process of excitotoxicity is now believed to include a series of genomic reactions from the expression of immediate early genes to the synthesis of proteins which in turn regulate the expression of other genes (Evan *et al.*, 1992; Smeyne *et al.*, 1993; Dave and Tortella, 1994; Lu *et al.*, 1997). In many cases the primary pathophysiological event associated with the initiation of neuronal injury is ischemia, or oxygen/energy deprivation leading to metabolic failure of the neuron and triggering EAA/glutamate release. Consequently, many reported studies have utilized various *in vitro* and *in vivo* models of pre- and post-synaptic hyperglutamatergic activity to study the molecular mechanism of neuronal injury.

Earlier studies from our laboratory and by others have implicated rapid changes in $[Ca^{2+}]_i$ dynamics as a seminal cause of EAA mediated neuronal death (Choi *et al.*, 1987; DeCoster *et al.*, 1994; Dave *et al.*, 1997). However, a precise balance of intracellular ions is crucial for the survival and recovery of an injured neuron, and hence, intracellular sodium dynamics and the state of Na^+-Ca^{2+} exchanger may be of equal importance. The Na^+-Ca^{2+} exchanger is considered one of the principal mechanisms by which neuronal $[Ca^{2+}]_i$ homeostasis is maintained (Sanchez-Armass and Blaustein, 1987; Blaustein, 1988), and several studies have proposed that the increase in intracellular calcium ($[Ca^{2+}]_i$) during hypoxia and ischemia is caused in large part by an increase in intracellular sodium $[Na^+]_i$ mediated by the reverse mode of Na^+-Ca^{2+} exchangers (Lysko *et al*, 1994; Calabresi *et al*, 1999). *In vitro*, anoxia has been reported to induce an increase in intracellular sodium levels leading to neurotoxicity, and the removal of extracellular Na^+ prevented anoxia-mediated morphological changes in hippocampal neurons (Friedman and Haddad, 1994). Furthermore, several *in vitro* and *in vivo* studies have demonstrated neuroprotective effects of use-dependent sodium channel blockers against ischemic/hypoxic injury (Lynch *et al*, 1995; Sun and Faden, 1995; Stys and Lesiuk, 1996; Campbell *et al*, 2000). Collectively, these studies suggest an important role of sodium channel mechanisms in neurodegeneration resulting from cellular ischemia/hypoxia.

The present studies were undertaken to determine the relative neuroprotective effects of RS-100642–198, a novel sodium-channel blocker that is structurally related to mexiletine, and two known, use-dependent sodium channel blockers (i.e. mexiletine and QX-314). The effects of these drugs were studied in three distinct *in vitro* neuronal injury models, namely, neurotoxicity induced by pre-synaptic glutamate release (i.e. hypoxia/hypoglycemia), post-synaptic glutamatergic (i.e. ligand gated) activity (i.e. glutamate), or the voltage-gated sodium channel activator veratridine. To understand, in a limited manner, the mechanism of action of these drugs, the excitotoxic dynamics of $[Ca^{2+}]_i$ was also studied using real-time laser cytometry in primary neurons depolarized by glutamate or KCl.

METHODS

Cell cultures

Adult time-pregnant Sprague-Dawley female rats were purchased from Taconic Farms (Germantown, NY) and enriched neuronal cultures were prepared from the brains of 17- day old rat embryos. Following euthanasia with carbon

dioxide, embryonic rats were removed from the uterus using aseptic techniques and placed in sterile neuronal culture media. Under a dissecting microscope, the brain tissue was removed from each embryo, taking care to discard meninges and blood vessels. The cerebellum was separated by gross dissection and cells were dissociated by trituration of the tissue and plated at a density of 5×10^5 cells/well in 48-well culture plates pre-coated with poly-L-lysine. Cultures were maintained in a medium containing equal parts of Eagle's basal medium (without glutamine) and Ham's F12 K media supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glucose (600 μg/ml), glutamine (100 μg/ml, penicillin (50 units/ml), and streptomycin (50 μg/ml). After 48 h, cytosine arabinoside (10 μM) was added to inhibit non-neuronal cell division. Our cultures typically yield 80–90% neurons and 10–20% glia and other cells (DeCoster *et al*, 1992; Ved *et al*, 1991).

Neurotoxicity experiments

Cells were used in experiments after 6–8 days in culture. Cells were exposed to glutamate (80 μM) or veratridine (20 μM) for 45 min in Locke's solution. H/H was induced by incubating the cells in a humidified airtight chamber saturated with 95% nitrogen:5% CO_2 gas for 2 hr in Locke's solution without added glucose. At the end of the treatment period ~~(45 min for glutamate or veratridine and 2 hr for H/H),~~ the Locke's solutions in each well were replaced with conditioned media (original) and morphological and cell viability (MTT measurements) assessments were made 24 hr later. Cell damage was quantitatively assessed using a tetrazolium salt colorimetric assay with 3-[4,5-dimethylthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chem. Co., Saint Louis, MO). Briefly, this dye was added to each well (final concentration of 0.15 mg/ml) and cells were incubated with MTT for 1 h at 37°C. When the assay was terminated, the dye was solubilized by acidified isopropanol (0.1 N HCl in isopropanol) and the absorbance intensity (540 nM) of each sample measured in a 96 well plate reader. In each toxicity model RS-100642–198 (0.1–500 μM), mexiletine (0.1–500 μM) or QX-314 (0.1–500 μM) were tested for neuroprotective potencies.

Values are expressed relative to vehicle-treated control cells (for glutamate or veratridine toxicity) that were maintained on each plate or relative to normoxic control cells maintained on a different plate, and percentage changes in cell viability calculated. Percent neuroprotection was calculated using the following formula in which "insult" refers to H/H, veratridine or glutamate treatments and "SCB" refers to a particular sodium channel blocker (either RS 100642, mexiletine or QX-314). Normoxic control values were used as "$\text{Survival}_{(\text{vehicle})}$" for H/H model in the following formula. Differences in the cell viability and % neuroprotection among treatment groups were determined using one-way analysis of variance and the Newman-Keuls Test.

$$\% \text{ Neuroprotection} = \frac{\text{Survival}_{(\text{insult+SCB})} - \text{Survival}_{(\text{insult})}}{\text{Survival}_{(\text{vehicle})} - \text{Survival}_{(\text{insult})}} \times 100$$

Measurement of intracellular free Ca^{2+} ($[Ca^{2+}]_i$)

Changes in $[Ca^{2+}]_i$ were determined using the fluorescent Ca^{2+}-sensitive dye fluo-3. Neurons were loaded with the membrane-permeable acetoxymethyl ester form of the dye by exposure to a reduced Ca^{2+} (0.2 mM) medium containing fluo-3-AM (5 μM) for 1 hr (37°C), then washed and maintained in Locke's solution at 37°C. Fluorescence changes in individual neurons were monitored using the InSight Plus confocal scanning laser microscope system (Meridian Instruments, Okemos, MI). Sequential image scans of fields containing 10–50 neurons (250×250 μm^2) were collected every 10 s to construct kinetic profiles of the effects of glutamate, KCl or ver-

atridine on $[Ca^{2+}]_i$ in the presence and absence of RS-100642–198, mexiletine or QX-314. A typical experiment lasts 500 sec and measurements ~~are~~ were made every 10 sec. Each experiment was begun with a 50–60 sec period of pre-experimental basal Ca^{2+} measurements followed by the addition of either a sodium channel blocker or vehicle (for another 40–50 sec), followed by the addition of glutamate, veratridine or KCl ~~and termination at 500 sec~~. To verify adequacy of dye loading and neuronal viability, the Ca^{2+} ionophore ionomycin (2 μM) was added 1–2 min before the end of each experiment. Neurons not responding or responding only very weakly to the treatment with ionomycin were not counted in these studies.

[^{3}H]-Batrachotoxin binding studies

Frozen brains from male Sprague-Dawley rats (200–400 g; Charles River) were obtained from Pel-Freez Biologicals (Rogers, AR). The cerebral cortices were removed and homogenized in 10 volumes of Tris-sucrose buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4 at 4°C) using 10 strokes with a glass-glass, hand-held homogenizer. The homogenates were filtered through nylon mesh and then centrifuged at 1000-x g for 15 min. The pellets were resuspended in a Hepes buffer (50 mM Hepes, 130 mM choline chloride, 5 mM glucose, 5.4 mM KCl, pH 7.4 at 25°C) and frozen at –70°C until use.

Rat cerebral cortical membranes were incubated with 16 nM [^{3}H]-batrachotoxin ([^{3}H]-BTX; Dupont-NEN, Boston, MA) in 250 μl of Hepes buffer (50 mM Hepes, 130 mM choline chloride, 5 mM glucose, 5.4 mM KCl, pH 7.4 at 25°C). Non-specific binding was defined in the presence of 1 mM veratridine. Membranes were incubated for 60 min at 25°C and then filtered using GF/B glass fiber filters that had been pre-treated with 0.3% polyethyleneimine (PEI). Filters were rinsed with 3 × 1 ml of ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C) and bound radioactivity was determined by scintillation counting (Packard Top-count). For data analysis, [^{3}H]-BTX inhibition curves were generated with 10 concentrations of inhibitor and analyzed by fitting data to a four-parameter logistic equation. IC_{50} values were then converted to K_I values using the Cheng-Prusoff equation.

Rat vagus nerve in vitro

Extracellular recordings were made of supramaximally stimulated C-fiber compound action potentials (CAPs) in rat vagus nerves in vitro, according to standard "grease-gap" techniques (Marsh *et al.*, 1987). Briefly, 2–3 cm lengths of vagus nerve were removed from male Sprague-Dawley rats (200–400 g; Charles River, MA) desheathed and placed in Marsh ganglion baths (Hugo Sachs Electronics, type 858). Nerves were bathed in oxygenated Krebs solution (following in mM: NaCl 119, KCl 2.5, $CaCl_2$ 2.0, $MgSO_4$ 1.3, $NaHCO_3$, 26.2, NaH_2PO_4 1.0 and glucose 11.0. The portion of the nerve in the center section of each bath was continuously superfused with control or drug-containing solution, heated to 34°C, at a flow rate of ~5 ml/min. A dedicated data acquisition system, using an analog-digital converter (Axon Instruments, Foster City, CA) displayed, stored and analyzed the AC voltage records. The acquisition program also directed a motorized valve (Hamilton Co., Reno, NV) to switch between solutions at set intervals. During each experiment, simultaneous recordings were made from up to four nerves. Cumulative concentration-inhibition curves for C-spikes were constructed using 10 min exposures to 1, 10, 100 and 1000 μM RS-100642–198 and mexiletine. Tonic block was measured with a slow rate of nerve stimulation (0.03 Hz) and phasic (or use-dependent block) with a 30-sec burst of 30 Hz stimulation at the end of each 10 min superfusion period. IC_{50} values for tonic and phasic inhibition were determined by SUNR techniques.

TABLE I *In vitro* Sodium Channel (NaCH) Properties of RS 100642–198

Assay	*RS 100642–198*	*Mexiletine*
[^{3}H]-Batrachotoxin[a]		
pK_i	5.09 [5.09–5.09]	4.94 [4.80–5.04]
Rat Vagus Nerve[b]		
Tonic IC_{50} (μM)	140 [120–160]	210 [160–280]
Phasic IC_{50} (μM)	130 [110–140]	200 [160–250]

a. [^{3}H]-batrachotoxin labeled sodium channels in rat cerebral cortex.
b. Use-dependent reduction in compound action potentials recorded from the vagus nerve (Tonic, 0.3 Hz; Phasic, 30 Hz).

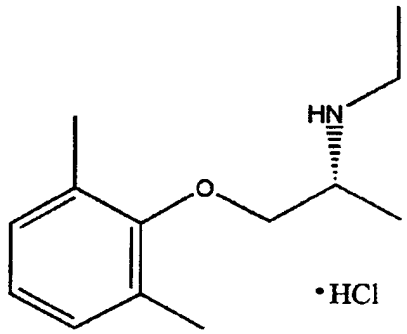
usually at the beginning of the methods section

Chemicals

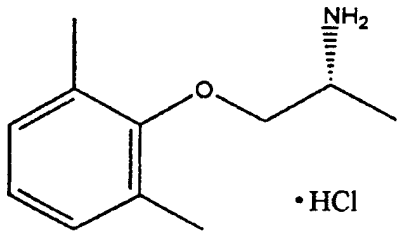
RS-100642–198 was synthesized at and obtained (along with mexiletine) from the Department of Medicinal Chemistry, Roche Bioscience (Palo Alto, CA). QX-314 was purchased from Alomone Labs (Jerusalem, Israel). As illustrated in Figure 1, both RS-100642–198 (1-(2′,6′-dimethyl-phenoxy)-2-ethylaminopropane hydrochloride) and mexiletine (1-(2′,6′-dimethylphenoxy)-2-aminopropane hydrochloride) are phenoxyaminopropanes and QX-314 (1-(2′,6′-dimethyl-phenylcarbamoylmethyl) triethylammonium bromide) is a phenylcarbamoyl compound. Veratridine and all other chemicals were of analytical grade and were purchased from the Sigma Chemical Co. (St. Louis, MO).

RESULTS

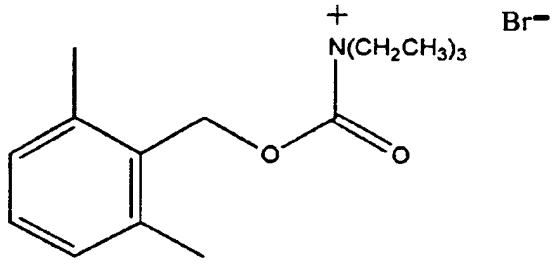
RS-100642–198 and mexiletine both produced a concentration-dependent inhibition of [^{3}H]-batrachotoxin labeled sodium channels present in rat cerebral cortical membranes (Table I). In addition, each compound, as expected, produced both a tonic and a small additional component of use-dependent inhibition of vagus nerve C-spikes *in vitro*. The K_i (radioligand binding) and IC_{50} values for the tonic:phasic components of the CAP (electrophysiology) are also displayed in Table I.



RS 100642-198



Mexiletine



QX-314

FIGURE 1 Chemical structures of two known, use-dependent sodium channel blockers (mexiletine and QX-314), and a novel sodium channel blocker, RS 100642–198

Figure 2 demonstrates the morphological changes observed in primary neurons exposed to vehicle/normoxic conditions (A), H/H (B), veratridine (20 μM; C) or glutamate (80 μM; D).

Shouldn't table 1 be after figure 1 (Fig 1 is mentioned first)

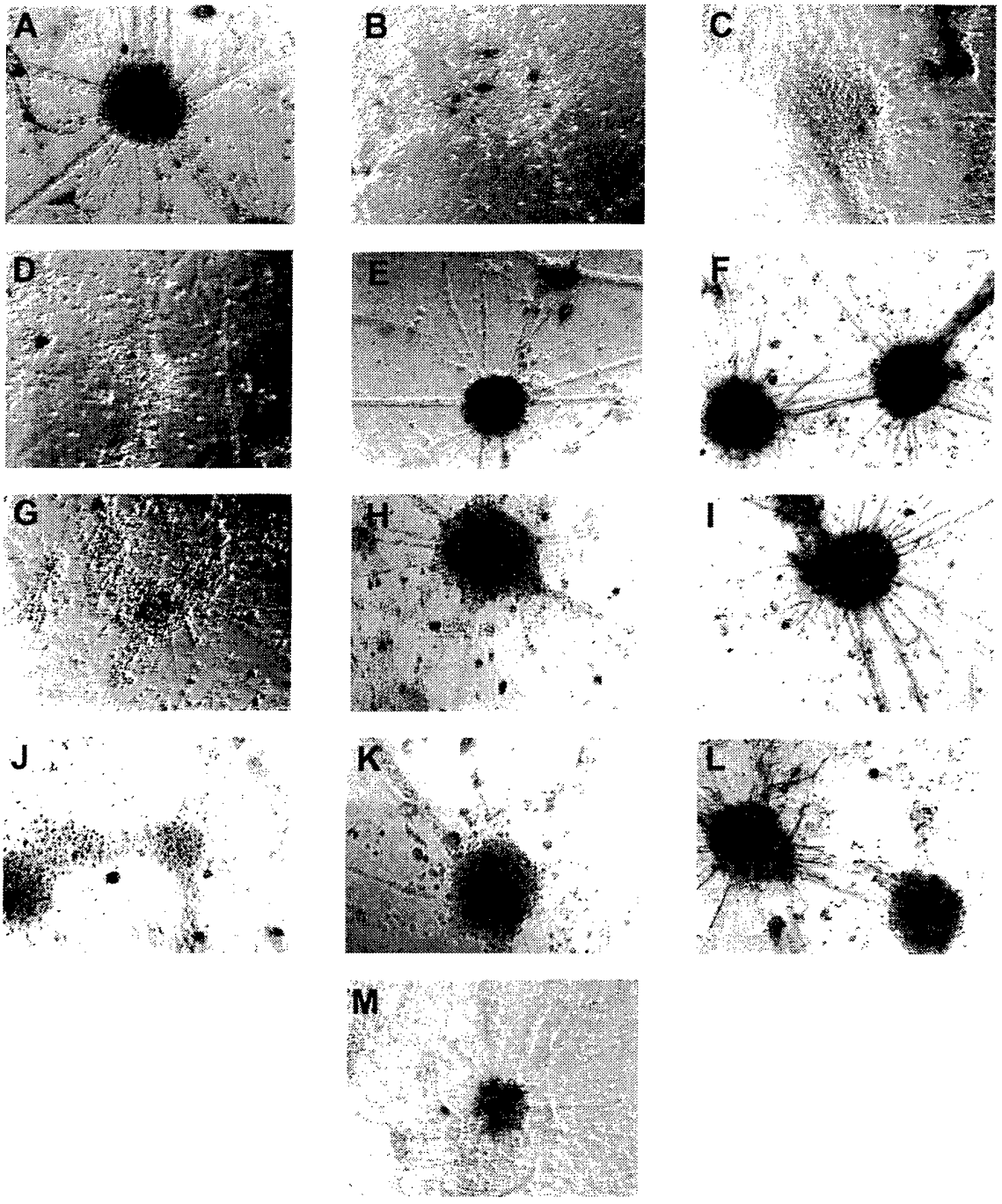


FIGURE 2 Representative bright-field micrographs (80x) of primary cultures of cerebellar neurons stained with MTT stain after either vehicle treatment (panel A) or H/H, veratridine (20 μM) or glutamate (80 μM) treated neurons (Panels B, C and D, respectively). Panels E, F and G: Neurons treated with 200 μM RS-100642–198 and subjected to H/H, veratridine or glutamate treatment, respectively. Panels H, I and J: Neurons treated with 200 μM mexiletine and subjected to H/H, veratridine or glutamate, respectively. Panels K, L and M: Neurons treated with 100 μM QX-314 and subjected to H/H, veratridine or glutamate treatment, respectively

should you mention that MTT "stains" viable (ie healthy) neurons?

In all the three models of neurotoxicity, cells lost their neuronal processes and shrank (Fig 2, B-D). Cell shrinkage was most pronounced following glutamate or veratridine treatment, otherwise the morphology of the respective injuries appeared similar. In general, in injured cells the number of neuronal processes or dendrites per neuron was also reduced compared to control/normoxic cells. In the veratridine-neurotoxicity model, the cells appeared to be more fragmented and often lost their round shape (Fig 2, C). Figure 2 (E-M) also shows the effect of RS-100642–198 (200 μM), mexiletine (200 μM) or QX-314 (100 μM) in neurons exposed to H/H, veratridine or glutamate. RS-100642–198 (Fig 2, E-G) or mexiletine (Fig 2, H-I) treatment prevented most of these morphological changes in H/H and veratridine-induced toxicity, although neither drug had a significant effect against glutamate-induced toxicity (Fig 2, G and J). QX-314, on the other hand, had a partial effect against H/H (Fig 2, K) and veratridine (Fig 2, L) neurotoxicity, but an even smaller effect on glutamate (Fig 2, M) mediated morphological changes. Cells treated with QX-314 appeared to have fewer but healthier neuronal processes, and cells bodies were less fragmented.

As shown in Figure 3, H/H, veratridine or glutamate exposures were highly neurotoxic to cerebellar neurons. In three separate experiments these treatments produced approximately 65%, 60% and 75% neuronal injury, respectively.

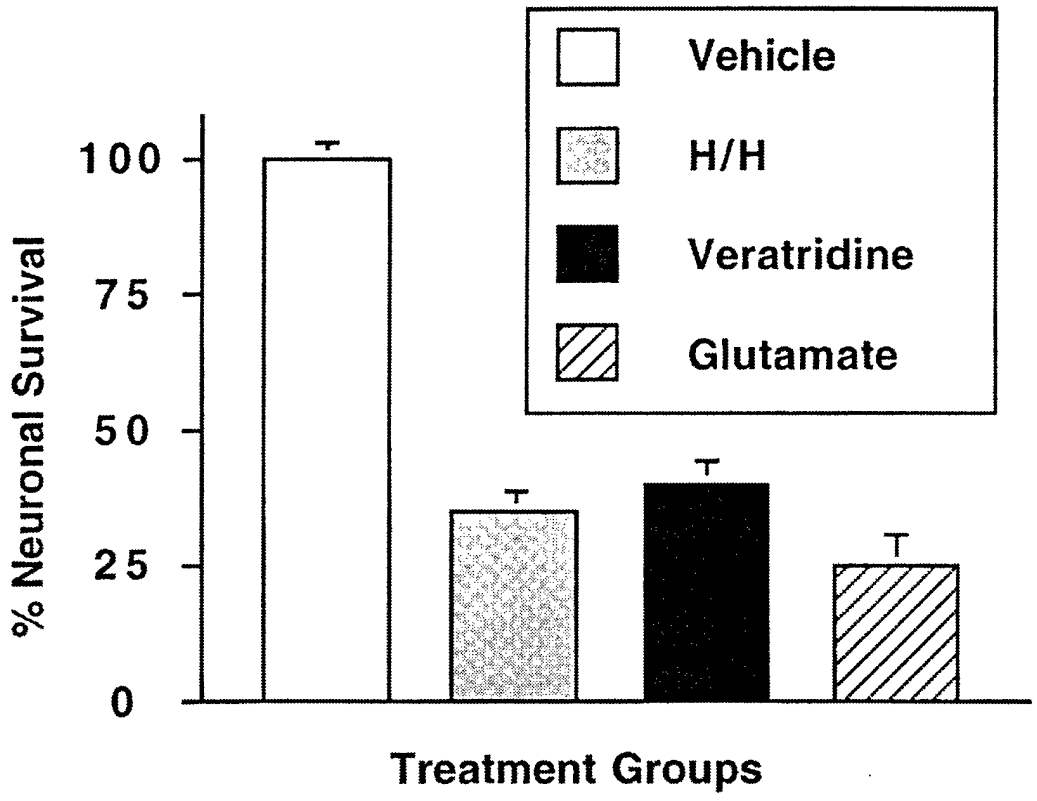


FIGURE 3 Demonstration that 2 hr of hypoxia/hypoglycemia (H/H), or exposure to veratridine (20 μM) or glutamate (80 μM) for 45 min produced significant neurotoxicity in primary cultures of cerebellar neurons. Values are mean ± SE of 4–6 determinations. All the three treatments groups are significantly different from vehicle control group at $p < 0.05$ (student's t-test). Similar results were obtained in at least 6 separate experiments

TABLE II Neuroprotective EC_{50}s (μM) and [95% CLs] of various sodium channel blockers against hypoxic/hypoglycemic (H/H), veratridine (20 μM) or glutamate (80 μM) insults

	H/H	*Veratridine*	*Glutamate*
RS-100642–198	60.8 [42.8–86.3] (76%)	6.9 [2.1–229] (100%)	No effect
Mexiletine	76.4 [58–101] (87%)	6.1 [2.7–13.9] (100%)	No effect
QX-314	144 [79.7–257.1] (66%)	12.1 [1.5–96.6] (50%)	11.5 [0.6–228] (25%)

Values in parenthesis are maximum neuroprotection observed.
Values are calculated from 4 separate experiments (n=12 wells/concentration).

RS-100642–198, mexiletine and QX-314 all produced a dose-dependent neuroprotection against H/H injury (Figure 4), with EC_{50} values of 61, 76 and 144 μM, and maximal efficacies of 76%, 87% and 66% neuroprotection, respectively (Table II).

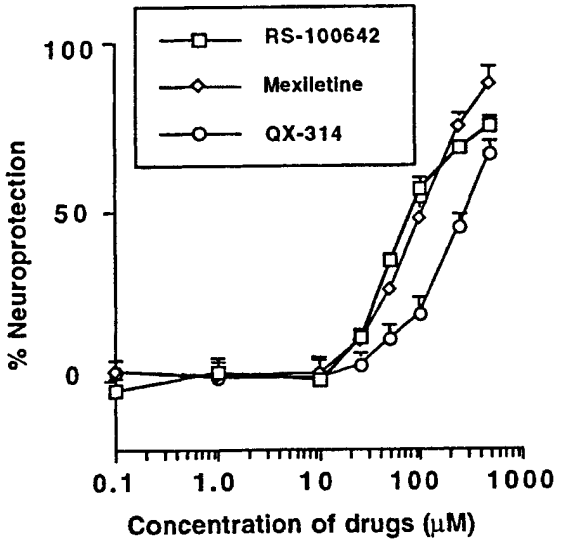


FIGURE 4 Representative dose-responses curve of neuroprotection by mexiletine, QX-314 and RS 100642–198 against hypoxia/hypoglycemia. Values are mean ± SE of 4 separate determinations. Similar results were obtained in at least two separate experiments

Against veratridine-induced injury RS-100642–198 and mexiletine were 100% efficacious while QX-314 neuroprotection was limited to (only) 54%; Fig 5). Importantly, RS-100642–198 and mexiletine exhibited significant neuroprotection at concentrations as low as 1 μM, whereas QX-314 neuroprotection was not significant until concentrations of 10–50 μM were used. The respective neuroprotection EC_{50}s for RS-100642–198, mexiletine and QX-314 were 7, 6 and 12 μM, respectively. These results are also summarized in Table II. Although these drugs by themselves did not produce cytotoxicity at concentrations up to 2 mM (data not presented), in the presence of veratridine both RS-100642–198 and mexiletine, at concentrations of 500 μM (Fig 5) and higher, were neurotoxic (data not presented).

In contrast to its neuroprotective effect against H/H and veratridine induced injury, RS-100642–198 was not neuroprotective against glutamate-induced injury at concentrations up to 500 μM. Similar results were seen with mexiletine. QX-314 produced a consistent, yet very limited (25%; range 19–28%), neuroprotection over a narrow dose-range of 50–200 μM. Interestingly, similar to veratridine induced neurotoxicity, RS-100642–198 and mexiletine were only cytotoxic when higher concentrations (> 100 μM) were tested with glutamate. These results are shown in Figure 6.

Neuronal $[Ca^{2+}]_i$ mobilization was measured in individual neurons and, consistent with prior observations (DeCoster et al., 1994), glutamate (80 μM) or KCl (25 mM) caused immediate and sustained increases in $[Ca^{2+}]_i$which remained elevated throughout the 500 sec observation period (Table III). A similar increase in $[Ca^{2+}]_i$was also observed with veratridine (20 μM) treatment. In these experiments, the basal/resting levels of $[Ca^{2+}]_i$ ranged from 70–355 nM. Additions of either glutamate, veratridine or KCl caused a peak increase in $[Ca^{2+}]_i$ to 1600–2800 nM (Table III). Post-peak levels of $[Ca^{2+}]_i$ ranged from 500–2400 nM. Glutamate

Table II after Fig. 4 ?
(it comes after in the text)

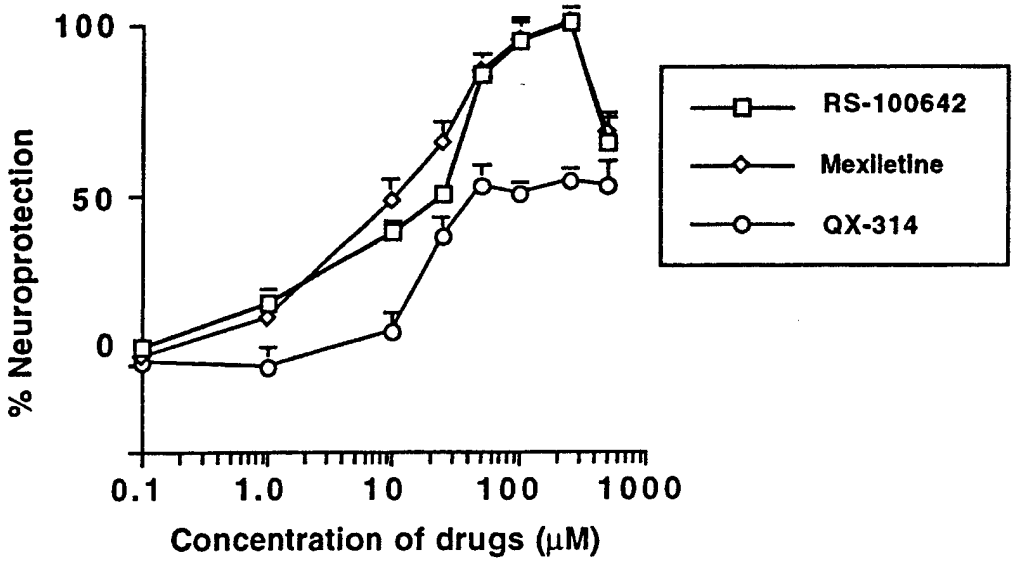


FIGURE 5 Representative dose-responses curve of neuroprotection by mexiletine, QX-314 and RS 100642–198 against veratridine. Values are mean ± SE of 4 separate determinations. Similar results were obtained in at least two separate experiments

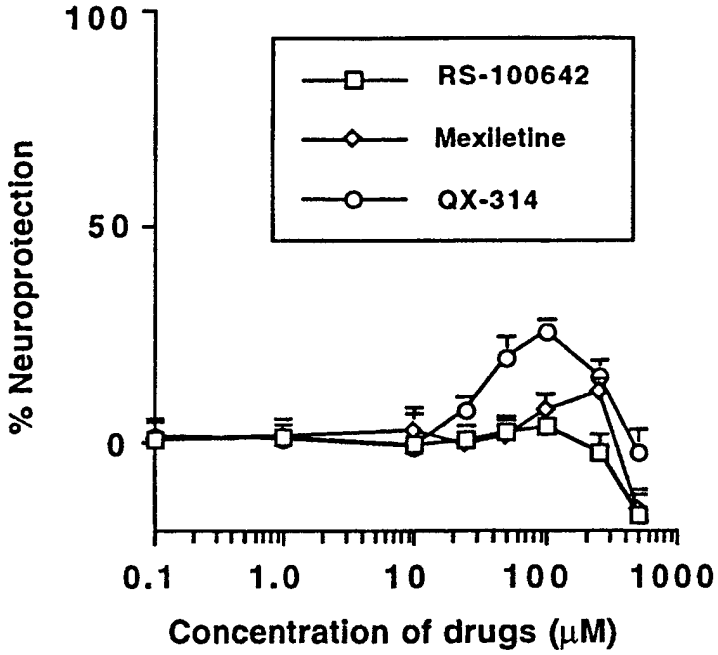


FIGURE 6 Representative dose-responses curve of neuroprotection by mexiletine, QX-314 and RS 100642–198 against glutamate. Values are mean ± SE of 4 separate determinations. Similar results were obtained in at least two separate experiments

caused the greatest increase in $[Ca^{2+}]_i$ (Peak = 2500–2800 nM; post-peak = 1800–2400 nM). Veratridine- and KCl-induced increases in $[Ca^{2+}]_i$ reached only to 1600–2000 nM (not as high as glutamate) at peak response, and decreased to a post-peak level of 500–900 nM. RS-100642–198 (or mexiletine) had no effect on glutamate-induced increase in $[Ca^{2+}]_i$. However, both drugs decreased the initial amplitude and attenuated the sustained rise caused by veratridine- or KCl-induced depolarization (Table III). In contrast, QX-314 produced similar effects on glutamate-, veratridine- or KCl-induced responses, effectively decreasing the amplitude and delaying the initial Ca^{2+} spike and attenuating the sustained increase in Ca^{2+} mobilization (Table III).

DISCUSSION

This study describes the neurotoxicity produced by H/H, veratridine and glutamate and demonstrates that the novel sodium channel blocker RS-100642–198 provides almost complete neuroprotection against H/H injury, or neuronal injury directly produced by sodium channel activation (e.g. by veratridine). However, RS-100642–198 failed to provide any significant neuroprotection against glutamate-mediated neurotoxicity. Similar results were obtained with mexiletine. By contrast, QX-314 was the only compound which provided limited (20–25%), but consistent, protection against neurotoxicity caused by glutamate.

As indicated earlier, a primary pathophysiological event associated with several types of neuronal injury is ischemia or tissue anoxia possibly leading to excitotoxic glutamate release. In this regard many studies have utilized various *in vitro* models of ischemia, e.g. hypoxia, hypoglycemia and cyanide exposure to study the molecular or cellular mechanisms of ischemia/EAA-mediated neuronal injury (Buisson and Choi, 1995; Lockhart *et al*, 1995; Lynch *et al*, 1995; Sun *et al*, 1997; Moro *et al*, 1998). Consistent with these models, we previously demonstrated that a combination of hypoxia and hypoglycemia for a period of 2 hr resulted in reproducible neuronal injury of approximately 60–70% (Lin *et al*, 1997), whereas hypoxia alone required a longer time period to induce a neuronal injury which was inconsistent and variable. Therefore, in the present study we utilized the combination of hypoxia and hypoglycemia *in vitro* to produce neuronal injury. Furthermore, along with the H/H model, we also used two additional excitotoxicity models to study the neuroprotective properties of RS-100642–198: glutamate-induced injury, a widely utilized neurotoxicity model and considered a "gold standard" in this research area, and veratridine-induced injury, a neurotoxicity model based on sodium channel opening-mediated depolarization.

TABLE III Effects of sodium channel blockers on mobilization of intracellular $[Ca^{2+}]_i$ following glutamate, KCl or veratridine treatment

Treatment		*Peak Ca^{2+}*	*Post-peak Ca^{2+}*
Glutamate	+ Vehicle	2718 ± 117	2213 ± 135
	+ RS 100642–198	2632 ± 105	2155 ± 186
	+ Mexiletine	2598 ± 143	2250 ± 166
	+ QX-314	1485 ± 133*	985 ± 125*
KCl	+ Vehicle	1816 ± 101	710 ± 76
	+ RS 100642–198	1292 ± 95*	221 ± 67*
	+ Mexiletine	1156 ± 76*	245 ± 38*
	+ QX-314	1386 ± 87*	341 ± 23*
Veratridine	+ Vehicle	1958 ± 132	896 ± 95
	+ RS 100642–198	1135 ± 105*	258 ± 89*
	+ Mexiletine	1048 ± 135*	189 ± 82*
	+ QX-314	1143 ± 137*	219 ± 44*

Values are intracellular calcium in nM and are mean ± SEM of 25–45 individual neurons. Values marked with an asterisk (*) are significantly different from vehicle control groups at $p < 0.05$ (Student's *t*-test). Similar results were obtained in two additional experiments. Concentrations of various compounds used were: 80 μM glutamate, 25 mM KCl, 20 μM veratridine, 200 μM RS 100642–198, 200 μM mexiletine and 200 μM QX-314.

Although the primary mechanism of action for H/H-mediated neuronal injury involves energy deprivation resulting in metabolic failure of the neuron, several studies have implicated activation of the glutamate/NMDA receptor complex as the imminent cause of neuronal death. (Lockhart *et al* 1995; Sun *et al*, 1997). However, a recent study has demonstrated that activation of the glutamate/NMDA receptor complex occurs secondarily in H/H injury, and injury is due to a primary activation of calcium and sodium channels leading to enhanced glutamate efflux into the extracellular space (Kimura *et al.*, 1998). Our present data also support such a hypothesis. Here both RS 100642–198 and mexiletine were equipotent, and exhibited comparable neuroprotection efficacies against H/H mediated neurotoxicity. However, both failed to provide any neuroprotection against glutamate-mediated neurotoxicity, indicating that H/H-mediated neuronal injury involves upstream mechanisms of excitotoxic neuronal injury, or modulation of sites on the post-synaptic membrane complex sensitive to sodium channel-induced depolarization.

The cytotoxic effects of glutamate have been demonstrated to be primarily as a result of an increase in intracellular calcium ion concentrations (Collingridge and Lester, 1989). Our earlier (Dave *et al*, 1997; De Coster *et al* 1994) and present findings support the above hypothesis and suggest a close correlation between neurotoxic and calcium-mobilizing effects of glutamate. Although the precise mechanism by which glutamate mediates neurotoxicity still remains to be fully defined, the initial blocking of neuronal calcium influx certainly provides significant neuroprotection (Choi *et al.*, 1987; DeCoster *et al.*, 1994; Dave *et al.*, 1997), suggesting a crucial role for Ca^{2+}-dependent cellular events. The present study demonstrates that the novel sodium channel blocker, RS-100642–198 failed to provide neuroprotection against glutamate toxicity and had no significant effect on glutamate-mediated increases in $[Ca^{2+}]_i$ However, it clearly attenuated both the veratridine- and KCl-mediated increases in $[Ca^{2+}]_i$. In this context, it has been suggested that, under ischemic conditions, blockade of sodium channels not only prevents the excessive depolarization and limits excitotoxic glutamate release (through reversal of the sodium-dependent glutamate transporter), but also allows calcium extrusion leading to reestablishment of the ionic homeostasis (Lasko *et al.*, 1994). Alternatively, under conditions of sustained Ca^{2+} influx, the Na^+-Ca^{2+} exchange system in primary neuronal cultures has been shown to manifest a Ca^{2+} extrusion system that attenuates delayed glutamate excitotoxicity (Andreeva *et al*, 1991). These data collectively support a key role for Na^+-Ca^{2+} exchanger system in the ischemic injury.

Veratridine-induced neurotoxicity primarily involves activation of voltage-dependent sodium channels leading to intracellular sodium ion overload within the neurons. One of the ways by which appropriate sodium and calcium ion concentrations are maintained within the cell is by the Na^+/Ca^+ exchanger. Any significant alterations in the concentrations of either calcium (e.g. by glutamate or hypoxia/hypoglycemia) or sodium (e.g. by veratridine or KCl) will result in an ionic imbalance within the neuron, and, if uncorrected, can result in cell death. Several *in vivo* studies have demonstrated rapid alterations in the distribution of ions across the cell membrane following CNS trauma. For example, in spinal cord injury extracellular levels of both Na^+ and Ca^{2+} at the site of impact decrease initially within the first few minutes only to be followed by a significant increase in these elements over a period of hours to days (Kwo *et al.*, 1989; Moriya *et al.*, 1994). Furthermore, exposure of cultured hippocampal neurons to glutamate has been reported to produce a significant increase in the cytosolic sodium concentration (Pinelis *et al.*, 1994). It is widely accepted that these ionic shifts initiate the progressive neuronal degeneration and loss of function that occur after CNS injury. It has been

reported that high concentrations of veratridine or KCl increase intracellular Na^+ in primary cultures of neurons, respectively, by activation of voltage-sensitive Na^+ channels or by directly opening these channels and by depolarization of the cell membrane leading to Na^+ influx, resulting in neuronal death (Takahashi *et al.*, 1999). Consistent with this are the neuroprotective effects of tetrodotoxin against veratridine-induced toxicity which have also been described in cultured rat cerebellar neurons (Lysko *et al.*, 1994). The present study demonstrates that the novel sodium channel blocker, RS-100642–198, and mexiletine provided almost complete neuroprotection against veratridine toxicity and suppressed both the KCl- and veratridine-mediated increases in $[Ca^{2+}]_i$. These results demonstrate the importance of intracellular Ca^{2+} dynamics in neuronal survival and further strengthen the hypothesis that voltage-sensitive Na^+ channels may play a key role in neurotoxicity and that blocking of these channels by appropriate Na^+ channel blockers, which also attenuate excitotoxic increases in $[Ca^{2+}]_i$, could provide complete neuroprotection.

Of three different sodium channel blockers studied, only QX-314 provided limited, but consistent, neuroprotection against a glutamate insult. Unlike RS-100642–198, QX-314 also produced an effect on glutamate-mediated Ca^{2+} mobilization, suppressing the immediate rises in $[Ca^{2+}]_i$ within 20–50 sec of the glutamate insult (albeit only at high [100–200 μM] concentrations). This partial suppression of glutamate-mediated increases in $[Ca^{2+}]_i$ by QX-314 compares favorably to its limited neuroprotection measured against glutamate injuries and was also seen following KCl or veratridine exposures. In contrast, in earlier experiments we have measured nearly complete suppression of glutamate-mediated increases in $[Ca^{2+}]_i$ by the non-competitive NMDA antagonist MK-801, which in turn also produced complete neuroprotection against glutamate toxicity (DeCoster *et al.*, 1994). The similar effects observed with QX-314 on KCl and veratridine suggest a non-selective blocking action of QX-314 on excitotoxic depolarizing responses. Alternatively, these limited actions of QX-314 on glutamate-mediated increases in $[Ca^{2+}]_i$ and neurotoxicity may be due to a sodium channel blocking action ~~from~~ at the "extracellular" membrane surface, an action not previously reported for this drug.

We have shown in this study differential effects of various sodium channel blockers on either hypoxia/hypoglycemia, glutamate or veratridine-mediated neurotoxicity. RS-100642–198 (and mexiletine) provided nearly complete neuroprotection against H/H or veratridine injury yet failed to protect against glutamate excitotoxicity, suggesting the possibility that in these injury models different types of sodium channels are being activated. Alternatively, intracellular Ca^{2+} mobilization may be the primary event driving glutamate neurotoxicity while activation of neuronal Na^+ channel occurs secondarily. This hypothesis is supported by Ca^{2+} mobilization data reported herein demonstrating that RS-100642–198 (and mexiletine) had no effect on glutamate-mediated increases in $[Ca^{2+}]_i$, but indeed suppressed KCl- or veratridine-mediated increases in $[Ca^{2+}]_i$. Consistent with this are the results of other studies describing similar actions where, for example, flunarizine was shown to provide complete neuroprotection against veratridine toxicity, but was ineffective against glutamate mediated neurotoxicity (Pauwels *et al.*, 1989). Furthermore, in cerebellar granule cells, the rate of $[Ca^{2+}]_i$ extrusion has been reported to be much slower following glutamate pulse than that after a comparable elevation in $[Ca^{2+}]_i$ induced by K^+ depolarization (Kiedrowski *et al.*, 1994). In this study, K^+ depolarization-mediated transient increase in $[Na^+]_i$ was much lower (from 4 mM to 13 mM) than that from glutamate (4 mM to 60 mM), further supporting the hypothesis that in these injury models probably different types of sodium channels may be activated.

How did you measure $[Na^+]_i$?
Or are you referring

Sodium channel blockers including lidocaine, QX-314, quinidine or lorcainide have also been shown to provide significant neuroprotection against H/H injury in cortical neurons but only when combined with other glutamate receptor antagonists (Lynch *et al.*, 1995). Other *in vivo* studies using sodium channel blockers such as tetrodotoxin and lamotrigine have described moderate neuroprotection in experimental models of brain ischemia (Lekieffre and Meldrum, 1993; Xie *et al.*, 1995; Kimura *et al*, 1998). Although lidocaine was reported to be ineffective in a cat model of focal cerebral ischemia (Shokunbi *et al.*, 1986), the sodium channel blocker and glutamate release inhibitor BW1003C87 has been shown to attenuate cerebral edema following experimental brain injury in rats (Okiyama *et al.*, 1995). The neuroprotective effects of tetrodotoxin in gerbil global brain ischemia has been documented by Lysko *et al* (1994), and in a recent preliminary study from our laboratory, RS-100642–198 treatment significantly reduced cerebral infarction resulting from middle cerebral artery occlusion (MCAo) (Williams *et al*, 1999).

Collectively, these studies support the theory that sodium channel blockers provide neuroprotection in both *in vitro* and *in vivo* experimental brain injury models and suggest a possible key role for selective types of sodium channels in peripheral and central nervous system related neuropathologies. The voltage-gated sodium channel comprises of α and β-subunits, with the pore forming α-subunit voltage-sensitive and ion specific. In different tissues and at different stages of development the α-subunit combines with a variable number of smaller β-subunits to form the bioactive channel (Isom *et al.*, 1992). The sodium channel α-subunits belong to a multigene family and cloning and electrophysiological characterization studies have documented the presence of at least eleven different sodium channel genes (e.g. rat brain I, II, IIA, III, rat Na6, PN1, PN3, NaG, rat SkM1, rat heart 1, NaN/SNS2) in the central and peripheral nervous systems and other excitable tissues (heart and muscle) in rodents (Gautron *et al.*, 1992; Schaller *et al.*, 1995; Waxman *et al.*, 1994; Akopian *et al.*, 1996; Sangmeswaran *et al.*, 1996; 1997; Novakovic *et al.*, 1998). In a recent preliminary study from our laboratory, MCAo for 2 hr followed by reperfusion in rats exhibited a time-related increase in rat brain type I (rBI) sodium channel expression (Cui *et al.*, 1999) suggesting the possible involvement of rBI neuronal sodium channel gene expression in CNS injury.

Development of selective blockers to target individual sodium channels and the role of sodium channels in neurodegeneration and repair has received limited attention. Although, the sodium channel blockers used in the present study are non-selective and probably do not target any single neuronal sodium channel, they have provided crucial information regarding efficacy and potency in different neurotoxicity models. In conclusion, our results demonstrate that the novel sodium channel blocker RS-100642–198 provided almost complete neuroprotection against neuronal injuries caused by cellular depolarization and sodium channel activation, but does not affect the ~~rather than~~ cellular consequences of post-synaptic glutamate hyperexcitability.

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Differential pattern of expression of voltage-gated sodium channel genes following ischemic brain injury in rats.

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Abstract

This study investigated the effects of brain ischemia on sodium channel gene (NaCh) expression in rats. Using quantitative RT-PCR, our findings demonstrated the expression ratio of NaCh genes in normal rat brain to be rBI>PN3>rBIII>PN1. In contrast, brain injury caused by middle cerebral artery occlusion (MCAo) for 2 h followed by reperfusion significantly down-regulated rBIII and PN1 genes in both injured and contralateral hemispheres; whereas the PN3 gene was down regulated in only the injured hemisphere (though only acutely at 2 or 2-6 h post-MCAo). However, the time-course of NaCh gene expression revealed a significant down-regulation of rBI only in the ischemic hemisphere beginning 6 h post-MCAo and measured out to 48 h post-MCAo. Our findings document, for the first time, quantitative and relative changes in the expression of various NaCh genes following ischemic brain injury and suggest that the rBI sodium channel gene may play a key role in ischemic injury/recovery.

Keywords: Ischemia, stroke, voltage-gated sodium channels, gene expression, quantitative RT-PCR.

Introduction

Cerebrovascular disease is a major cause of death and disability (Bonita, 1992) caused by the metabolic failure of neurons and glia due to deprivation of continuous supply of oxygenated blood. Insults such as stroke or ischemia are known to trigger a vicious cycle of electrical and chemical events (Lee *et al.,* 1999; Carter, 1998). It is now widely accepted that neuronal cell death caused by ischemia results from a cascade of events, ranging from excessive presynaptic release of excitatory amino acids (EAA), alterations in cellular ionic dynamics, toxic postsynaptic accumulation of intracellular calcium $[Ca^{2+}]_i$, and ultimately the activation of secondary signaling mechanisms leading to acute or delayed injury processes (Choi, 1987; DeCoster *et al.*, 1992; Mattson *et al.*, 1992). This process of excitotoxicity is also believed to include a series of genomic events from the expression of immediate early genes to the synthesis of proteins, which in turn regulate the expression of other genes (Evan *et al.*, 1992; Smeyne *et al.*, 1993; Dave and Tortella, 1994; Lu *et al.*, 1997).

Voltage-dependent sodium channels (NaChs) may play a crucial role in neuron excitability and are considered one of the several cellular targets for neuroprotective mechanisms of action. It has been suggested that under ischemic/excitotoxic conditions blockade of sodium channels not only prevents the excessive depolarization and limits excitotoxic glutamate release (through reversal of the sodium-dependent glutamate transporter), but also allows calcium extrusion leading to reestablishment of the ionic homeostasis (Lysko *et al.*, 1994). In fact, several *in vivo* studies using sodium channel

blockers such as tetrodotoxin and lamotrigine have described moderate neuroprotection in experimental models of brain ischemia (Lekieffre and Meldrum, 1993; Lysko *et al.*, 1994; Xie *et al.*, 1995; Kimura *et al*, 1998), and in a recent preliminary study from our laboratory, a novel NaCh blocker RS-100642-198 was described to significantly reduce cerebral infarction resulting from MCAo (Williams *et al*, 1999). As such, changes in the transcription of NaChs genes could be involved in the neuronal injury associated with brain ischemia, as well as the neuronal plasticity associated with functional recovery.

The sodium channel comprises of α and β-subunits, with the pore forming α-subunit voltage-sensitive and ion specific. In different tissues and at different stages of development the α-subunit combines with a variable number of smaller β-subunits to form the bioactive channel (Isom *et al.*, 1992). The sodium channel α-subunits belong to a multigene family and cloning and electrophysiological characterization studies have documented the presence of at least eleven different sodium channel genes (e.g. rat brain I, II, IIA, III, rat Na6, PN1, PN3, NaG, rat SkM1, rat heart 1, NaN/SNS2) in the central and peripheral nervous systems and other excitable tissues (heart and muscle) in rodents (*Noda et al.*, 1986; Auld *et al*, 1988; Kayano *et al*, 1988; Gautron *et al.*, 1992; Waxman *et al.*, 1994; Schaller *et al.*, 1995; Akopian *et al.*, 1996; Sangameswaran *et al.*, 1996; 1997; Novakovic *et al.*, 1998). However, the specific physiological roles distinguishing each of these isoforms remain unknown. One possible explanation for this is that the differences in the functional properties of these channels are subtle (Noda *et al.*, 1986; Auld *et al.*, 1998). Nevertheless, such subtle differences could be functionally important, because small changes in voltage dependence of activation or inactivation could markedly affect excitability.

In order to assess the relationship between differential gene expression and function, it is essential to accurately and quantitatively measure mRNA levels. While a number of techniques, such as Northern blotting and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) are available to measure levels of mRNA expression, certain limitations exist, including the insensitive and potentially inaccurate quantitation of mRNA that may be normally expressed in low abundance. Indeed, using these non-quantitative techniques our initial pilot studies revealed highly variable results in NaCh gene expression in injured rat brain (Cui *et al.*, 1999). Recently described methods for quantitative RT-PCR are more sensitive, more accurate and able to detect and amplify low abundance mRNA (Livak *et al.*, 1995; Gibson *et al.*, 1996). Using quantitative RT-PCR, the present studies were therefore undertaken to determine the time-related changes in expression of four neuronal sodium channel genes, namely rat brain sodium channel type I and III (rBI and rBIII) and peripheral nerve type I and III (PN1 and PN3) in rat brain following focal brain ischemia produced by MCAo.

Materials and methods

1. Focal ischemic surgery

Middle cerebral artery occlusion was carried out according to our previously described method (Phillips *et al.*, 2000; Williams *et al.*, 2000). Briefly, male Sprague-Dawley rats weighing 270-330 g (Charles River Labs, Raleigh, VA) were anesthetized by 5% halothane and maintained at 2% halothane. An intraluminal filament occluded the right middle cerebral artery for a period of 2 h followed by reperfusion for 0, 4, 22 and 46 h. Control groups of rats received sham surgery, in which an identical procedure was followed but without inserting the filament (no occlusion). From each injured brain a 2 mm thick coronal slice located 5 mm from the frontal pole was dissected for RNA extraction. From this slice injured and contralateral hemispheres were dissected out. The remaining brain tissue was stained with 2, 3, 5-triphenyltetrazolium chloride (TTC) for infarct analysis (see Williams *et al.*, 2000) in order to ascertain that the MCAo was successful.

2. Total RNA isolation

The tissue samples were homogenized in TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). Total RNA was extracted from the tissue according to the manufacturer's suggested protocol. The total RNA concentration was determined by spectrophotometry at the absorbency 260 and 280 nm.

3. Primers and probes

The primers and probes for sodium channel genes rBI, rBIII, PN1, PN3 and house keeping gene β-actin were designed using the primer design software Primer Express ™ and their sequences are presented in Table 1. Synthesis of these probes and primers was performed by Perkin-Elmer Applied Biosystems Foster City, CA. FAM (6-carboxyfluorescein) was used as the reporter and TAMRA (6-carboxy-tetramethyl-rhodamine) as the quencher dye. The housekeeping gene β-actin was used as an endogenous control in these samples to provide sample amplification efficiency.

4. Quantitative RT-PCR reaction

RT and PCR were carried out using a GeneAmp RNA PCR Core Kit and TaqMan Universal PCR Master Mix kit (Perkin-Elmer) according to the manufacturer's specification. A two-step RT-PCR was performed. The RT reaction used 10 μg total RNA in a total volume of 100 μl containing 1x PCR Buffer II, 5 mmol/L $MgCl_2$, 1mmol/L of each dNTP, 2.5 μmol/L Random Hexamers, 1 U/μl RNase Inhibitor and MultiScribe Reverse Transcriptase. The RT reaction was carried out at 42°C for 15 min, 99°C for 5 min. The second cDNA synthesis and quantitative PCR were performed in the TaqMan Universal PCR Master Mix with 5-10 μl of each RT product (rBI 5 μl, rBII 8 μl, PN1 10 μl and PN3 8 μl; 1 μl of RT products contains 0.1 μg total RNA), 100 nmol/L probe and 200 nmol/L primers in a total volume of 50 μl. PCR was performed at 50°C

for 2 min, at 95°C for 10 min and then run for 40 cycles at 95°C for 15 seconds and again at 60 °C for 1 min on the ABI PRISM 7700 Detection System. A single specific DNA band for rBI, rBIII, PN1 and PN3 was observed on Southern gel electrophoresis analysis (data not shown). Using the formula provided by the manufacturer (Perkin-Elmer) and also described by Wang *et al.*, 2000; the values were extrapolated to calculate the relative number of mRNA copies.

5. Statistical analysis.

Data are presented as mean ± s.e.m. Statistical comparisons (n = 8/group) were made by analysis of variance (ANOVA; Fisher's protected least squares difference) and values were considered to be significant when $P < 0.05$.

Results

1. Infarct analysis

All rats survived throughout the experiment. Computer-assisted image analysis was used to digitally image the posterior surface of each TTC-stained forebrain section (Loats Associates, Westminster, MD). Figure 1 show the representative images of coronal sections demonstrating ipsilateral infarcts in the ischemic hemisphere, and the corresponding non-infarcted contralateral hemisphere, taken from animals at 2 h to 48 h post-MCAo. At 2 h post-MCAo (no reperfusion) there was no obvious ischemic damage to the cerebral tissue. The severity of the ischemic injury was progressive as the reperfusion time extended from 6 h to 48 h post-MCAo.

2. Standard curves

Calibration curves for quantification of sodium channel gene expression were constructed on a 1:2 serial dilution of DNA Template Reagents (Perkin Elmer) (Figure 2; Top). These standard curves demonstrate that quantitation of each target gene was linear on a scale of at least 7 orders of magnitude with excellent correlation factors of 0.99 and slope values ranged between 3.6-4.2 (Figure 2; Bottom).

3. NaCh gene expression efficiency in normal rat brain

Quantification of the level of relative mRNA for each sodium channel gene was first analyzed in normal rat brain. The mRNA levels of rBI, rBIII, PN1, PN3 and the house-keeping gene β-actin detected by quantitative RT-PCR and shown in Figure 3 demonstrate that rBI is the most abundant of the four sodium channel genes expressed in normal rat brain. Compared to β-actin gene expression, the relative level of expression of each of the NaCh genes were rBI, 18%; rBIII, 8.3%; PN1, 7.3% and PN3, 9.6%. The rBI gene expression was significantly greater than the other NaCh genes, whereas the differences between these channels (i.e. rBIII, PN1 and PN3) were very small and not significant.

4. NaCh gene expression during MCAo injury

NaCh gene expression was quantified at various time points following MCAo and the results are shown in Figure 4 (A-D). There was no significant difference in rBI gene expression in the contralateral (i.e. uninjured) hemisphere (compared to the same hemisphere from sham controls) at any time point studied. However, a significant down-regulation of rBI gene expression was detected in the ischemic hemisphere from 6 h post-MCAo to 48 h post-MCAo with a maximal decrease being observed at 24 h post-injury (Fig 4A).

Compared to sham controls, rBIII and PN1 genes were significantly down regulated in both injured and contralateral hemispheres (though only acutely at 2 or 2-6 hr post-MCAo). The PN3 gene was significantly down regulated in only the injured

hemisphere at 2-6 h post-MCAo (Fig 4B-4D). However, there was no significant difference in the expression levels of these three NaCh genes at any time in the injured hemisphere when compared with their contralateral hemispheres.

Discussion

The present study describes changes in brain type (rBI and rBIII) and peripheral nerve type (PN1 and PN3) sodium channel gene expression using quantitative RT-PCR in the MCAo model of rat brain injury. Although expression of peripheral nerve type sodium channel PN1 has been documented in normal rat brain (Sangameswaran *et al.*, 1997), to our knowledge, this is the first time that PN3 expression has been demonstrated in normal or injured rat brain. Furthermore, the significance of this study is the fact that this is the first and only demonstration of changes in expression of NaCh genes following ischemic brain injury, in particular the regionally selective down-regulation measured for the rBI gene.

The application of quantitative RT-PCR was critical to our success in studying NaCh gene expression in rat brain since unlike other gene families such as early immediate genes, the abundance of normal endogenous NaCh mRNA is low (Dib-Haji *et al.*, 1996; Sangameswaran *et al.*, 1997; Waxman *et al.*, 2000) and the turnover of functional channels is relatively very slow (Waechter *et al.*, 1983; Schmidt and Catterall, 1986). In fact, as noted earlier our pilot studies using non-quantitative RT-PCR or Northern hybridization exhibited results of NaCh gene expression in injured rat brain which while demonstrating similar trends to those reported here, were highly variable and statistically non-significant (Cui *et al.*, 1999).

In the present study, we demonstrated that among the four sodium channel genes studied, the abundance of rBI channel mRNA in normal rat brain is maximum, followed

by PN3, rBIII and PN1 mRNA. These results differ slightly from those reported earlier in rat dorsal root ganglia (DRG) by Sangameswaran *et al*, (1997) where rBI expression was maximum, followed by PN1, PN3 and only a minimum of rBIII expression. Although it is not surprising that the order of NaCh genes expression in the present study (i.e. brain neurons) and that reported by Sangameswaran and co-workers (i.e. peripheral DRG neurons) appears different, it is surprising that in both studies it has now been shown that the rBI gene was the most prominent NaCh gene expressed. From these collective results it appears that the rBI NaCh may be one of the most prominent ion channels expressed in both central and peripheral nervous systems. Of course, translation of rBI mRNA into an active channel remains to be established. Furthermore, although expression of the PN1 gene in normal brain tissue has been previously demonstrated by the RT-PCR method (Sangameswaran *et al.*, 1997), our report is the only study describing significant levels of PN3 gene expression in both normal and injured rat brain. This discovery could simply reflect the highly sensitive quantitative RT-PCR method used in the present study, as other investigators (Novakovic *et al.*, 1998) have also failed to detect PN3 mRNA in rat brain using *in situ* hybridization technique. In this context it is worth mentioning that using *in situ* hybridization technique (Felts *et al.*, 1997) also failed to observe the presence of PN1 mRNA in either hippocampus, cerebellum and spinal cord during development (E17-P30) in rats.

The time-course of changes in rBI gene expression in our study revealed a significant down-regulation in the ischemic hemisphere from 6 h to 48 h post-MCAo with a maximal decrease being observed at 24 h post-injury. Similarly PN3 NaCh gene

was also down regulated in injured hemisphere, though only acutely. Compared to sham controls, rBIII and PN1 genes were also significantly down regulated, but this was seen in both injured and contralateral hemispheres (though acutely at 2 or 2-6 h post-MCAo). However, when compared with their contralateral hemispheres, there was no significant difference in the expression levels of these genes (i.e. rBIII, PN1 and PN3) in the injured hemisphere at any time interval studied. In view of the fact that there is a significant neuroanatomical and functional link between the brain hemispheres, one might expect profound cellular changes in the contralateral hemisphere of the brain following ischemic insult. However our results show that rBIII, PN1 and PN3 sodium channel genes did not exhibit significant difference between the hemispheres, but rather only an acute reduction in the expression of these NaCh genes in both the hemispheres. Though speculative, it is possible that the contralateral hemisphere may have enough uncompromised sodium channel on the neuronal plasma membrane that the signaling mechanism to activate or inhibit NaCh gene expression may not have triggered. In fact in a recent EEG topography study in a similar MCAo injury model, we observed that EEG activity in the contralateral hemisphere was also altered as intermittent rhythmic activities (4-6 Hz) were observed in the frontal and central parietal regions, and increased beta activity in the temporal cortical regions, of the contralateral hemisphere (Lu *et al.*, unpublished observations). These results indicate that the ischemic insult also affects neuronal activity (and likely membrane conductance) in the contralateral hemisphere. In this regard, the rBI gene appears to be the only sodium channel gene studied to date which is truly down-regulated in a time-dependent manner following ischemic insult in MCAo brain injury model.

Gene expression following cerebral ischemia involves the up-regulation of several gene families which are involved in the progression of brain injury. These genes include immediate early genes, heat shock proteins, cytokines, adhesion molecules, and apoptotic genes. Glutamate-induced calcium influx due to cerebral ischemia is considered to be one of the prominent mediators of this genomic response (Savitz and Rosenbaum, 1999). However, inn some cases down-regulation of possible signaling mechanisms, such as excitatory amino acid (EAA) receptors and voltage-gated ion channels, has also been shown to change following brain injury and it is possible that this post-ischemic target receptor down-regulation may represent an endogenous protection mechanism of cells to reduce EAA excitotoxicity. Examples include the reduction of NR2A, NR2B, GLUR2 and GLUR3 in the hippocampal CA1 region of the rat following global ischemia (Zhang *et al.*, 1997; Pellegrini-Giampietro *et al.*, 1994). Also, in primary neuronal cultures from rats scorpion neurotoxin [a sodium channel (NaCh) agonist] decreased the expression of rBI, rBII, and rBIII (Lara *et al.*, 1996) and in rats with kainite-induced seizures, the down-regulation of rBII and rBIII (adult subtype) NaCh gene expression in the hippocampus has also been documented (Gastaldi *et al*, 1997), indicating that persistent activation may relate to a molecular and functional down-regulation of these channels. Importantly, this down-regulation has also shown to be sensitive to the calcium-dependent CaM kinase II activation (Carlier *et al.*, 2000). Thus, the increases in intracellular calcium following brain ischemia may induce signaling cascades involved in altering the expression of NaChs. Finally, as an interesting footnote it has also been reported that diving turtles exhibit reduced NaCh expression during hypoxic periods, possibly as a protective adaptation strategy (Perez-Pinzon *et al.*, 1992).

Chronic receptor stimulation during brain injury may be due to increased glutamate levels, which is involved in excitotoxic injury (Lipton and Rosenberg, 1994). Our data has confirmed that the down regulation of several NaCh genes does in fact occur following focal cerebral ischemia. Importantly, the down regulation observed in glutamate receptors (Lipton and Rosenberg, 1994) as well as that measured for voltage-gated NaChs in the present study, was delayed post-injury (i.e. > 2-6 h post-injury). This becomes important in future drug development studies, which may target specific receptor subunits. If the target receptor is being down-regulated, efficacy of drug treatment targeting these receptors may be lost. Thus, studying the time course of gene expression of excitatory amino acid and voltage-gated ion channels is a crucial direction for future research consistent with advanced drug development targets.

In conclusion, the present study reports expression of both brain type and peripheral type sodium channels in uninjured and injured rat brain. We have also demonstrated time-dependent changes in the expression of these four sodium channel genes following MCAo injury. Although, rBIII, PN1, PN3 gene expression did not significantly differ (compared to contralateral hemisphere) following MCAo injury, rBI gene expression showed a clear and dramatic time-related decrease. The reason for this decrease in rBI gene expression following MCAo injury remains speculative. In view of the fact that sodium channel blockers have been shown to be neuroprotective against ischemic insult (Ashton *et al.*, 1997; Dave *et al.*, 2001) it is possible that vulnerable neurons may reduce the availability of rBI sodium channel protein (by decreasing its expression) as a defense mechanism against further or delayed neurodegeneration. Although the present study suggests a possible involvement of the rBI sodium channel

gene in the injury and/or recovery process further studies are needed, possibly using antisense oligonucleotides, to specifically evaluate the role of each NaCh subunit during the injury/recovery process.

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Figure legends

Figure 1. Representative forebrain images showing injury at different time after MCAo. 2 mm thick brain sections were stained with TTC as described earlier.

Figure 2:

Upper panel: A representative standard curve showing real-time amplification plots of the standard DNA.

Lower panel: A representative standard curve showing linear correlation between threshold cycle and the initial amount of copy numbers.

Figure 3 Relative expression efficiency of four sodium channel genes and β-actin gene. Values are mean ± S.E. of 8 separate brain samples. Values marked with an asterisk (for rBI) are significantly different from rBIII, PN1 and PN3 values.

Figure 4 Time course of sodium channel rBI (Panel A), rBIII (Panel B), PN1 (Panel C) and PN3 (Panel D) genes expression following MCAo injury. Open bars represent values for contralateral hemispheres and filled bars represent values from injured (ipsilateral) hemispheres. Values are mean ± S.E. of 8 separate brain samples. RT products used for PCR were rBI 5 μl, rBII 8 μl, PN1 10 μl and PN3 8 μl (1 μl of RT products contains 0.1 μg total RNA). Values marked with a plus (+) sign are significantly different from the sham control values and those marked with an asterisk (*) are

significantly different from the values for their respective contralateral hemispheres at $p < 0.05$.

Table 1. The sequences of forward and reverse primers and probes used for quantitative RT-PCR of four sodium channel genes and β-actin gene expression. The probes consisted of a reporter dye (6-carboxyfluorescein; FAM) at 5' position and a quencher dye (6-carboxy-tetramethyl-rhodamine; TAMRA) at 3' position.

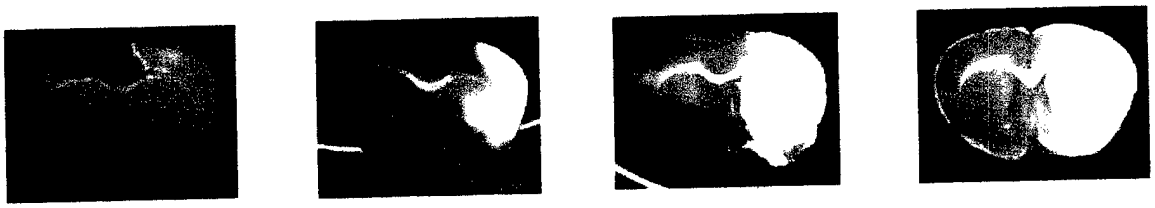

Figure 1

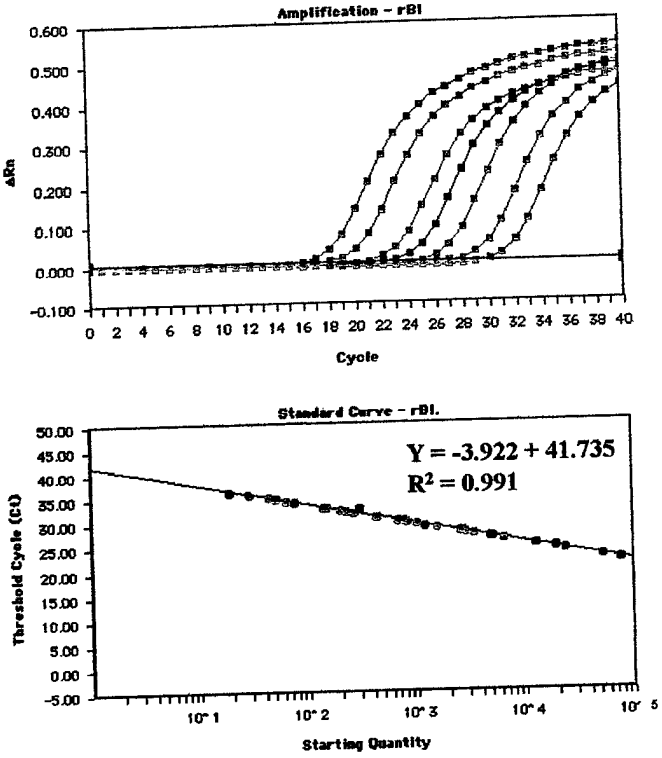
Amplification - rBI
ΔRn
0.600
0.500
0.400
0.300
0.200
0.100
0.000
-0.100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle
Standard Curve - rBI.
Y = -3.922 + 41.735
R² = 0.991
Threshold Cycle (Ct)
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
10^1
10^2
10^3
10^4
10^5
Starting Quantity

Figure 2

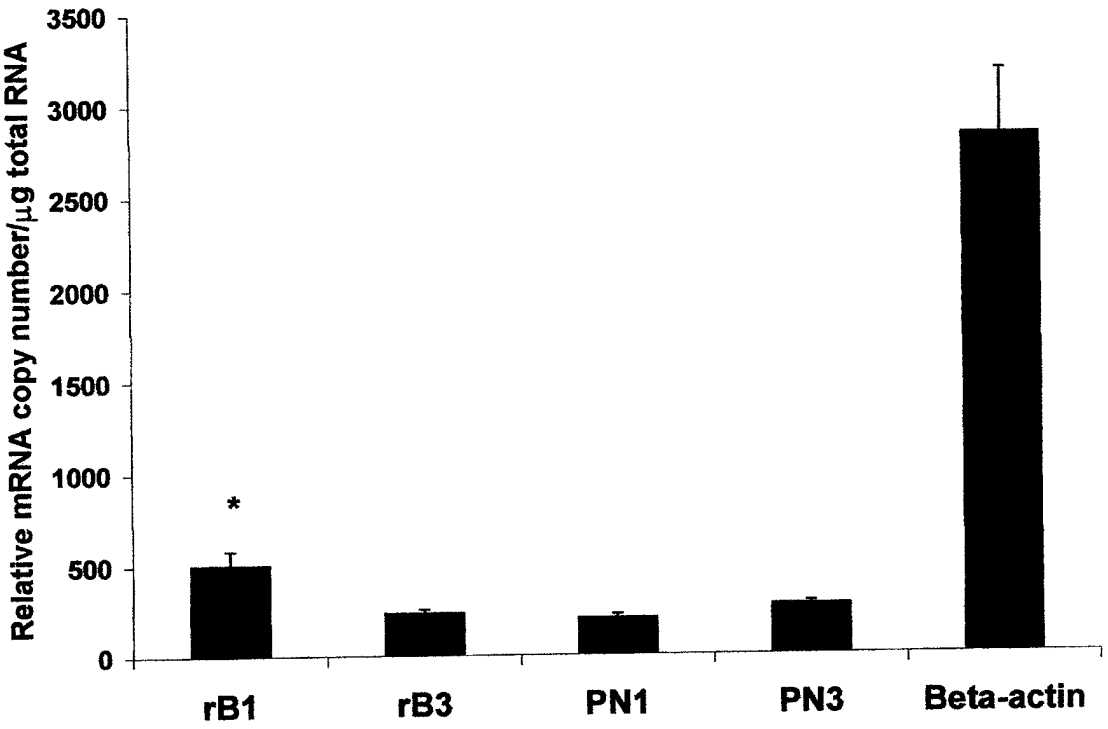
Relative mRNA copy number/μg total RNA
3500
3000
2500
2000
1500
1000
500
0
*
rB1
rB3
PN1
PN3
Beta-actin

Figure 3

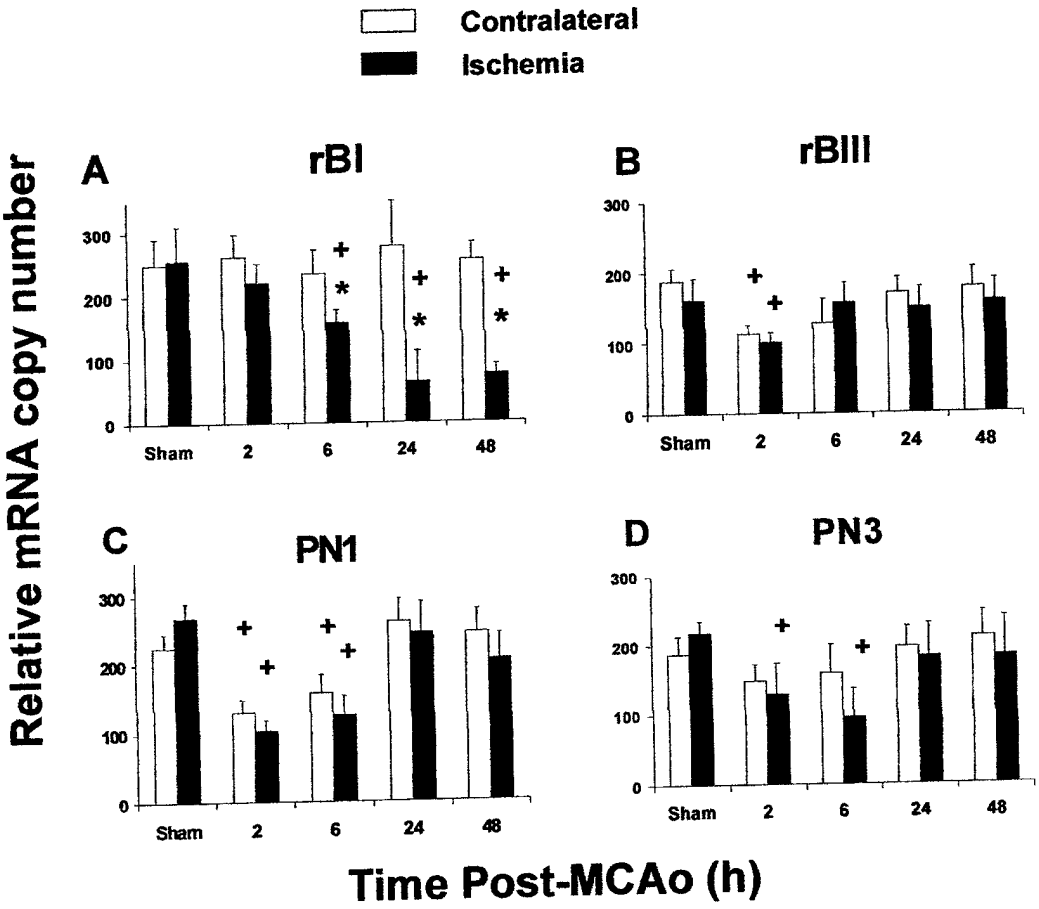
Contralateral
Ischemia
A
rBI
B
rBIII
C
PN1
D
PN3
Relative mRNA copy number
Sham
2
6
24
48
Time Post-MCAo (h)

Figure 4

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Neuroprotective effects of the sodium channel blocker RS100642 and attenuation of ischemia-induced brain seizures in the rat

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Abstract

Seizurogenic activity develops in many patients following brain injury and may be involved in the pathophysiological effects of brain trauma and stroke. We have evaluated the effects of the use-dependent sodium channel blocker RS100642, an analog of mexiletine, as a neuroprotectant and anti-seizure agent in a rat model of transient middle cerebral artery occlusion (MCAo). Post-injury treatment with RS100642 (0.01-5.0 mg/kg) dose-dependently reduced brain infarction, improved functional recovery of electroencephalographic (EEG) power, and improved neurological outcome following 2 h of MCAo and 24 h recovery. This effect was more potent and offered a larger reduction of brain infarct volume than a maximal neuroprotective dose of mexiletine (10.0 mg/kg). Furthermore, brain seizure activity recorded following 1 h MCAo and 72 h of recovery in injured rats was either completely blocked (30 min pre-MCAo treatment) or significantly reduced (30 min post-MCAo treatment) with RS100642 (1.0 mg/kg) treatment resulting in greater than 60% reduction of core brain infarct. These results indicate that brain seizure activity during MCAo likely contributes to the pathophysiology of brain injury and that RS100642 may be an effective neuroprotective treatment not only to decrease brain injury but also to reduce the pathological EEG associated with focal ischemia.

Key words: brain injury, MCAo, sodium channels, seizures, neuroprotection

1. Introduction

Thromboembolic stroke is the third leading cause of death in the United States and Europe as well as producing functional disability among survivors [5]. Furthermore, seizures have been reported to occur in up to 50% of brain injured patients depending on the type of injury induced, including traumatic brain injury, hemorrhage, or stroke [14,27,35]. Anticonvulsant therapy with agents possessing sodium channel blocking properties is currently available for the treatment of seizures (i.e. phenytoin and carbamezapine) but to date the prophylactic use of anticonvulsants following brain ischemia is not widely practiced except in those patients who develop overt convulsant seizure activity [2]. Recently EEG studies of brain injured patients have indicated that the presence of seizure activity is actually higher than previously estimated due to the "nonconvulsant" nature of post-traumatic brain seizures [35].

The normally functioning brain is solely dependent upon blood-delivered glucose as an energy source for the production of adenosine-triphosphate (ATP). Positron emission tomography (PET) studies in humans during epileptic seizures have indicated an acute rise in glucose utilization occurring during ictal activity [21]. Consequently, following a cerebral ischemic episode, seizure-induced increases in glucose metabolism could rapidly deplete cellular energy stores in the ischemic regions. Upon depletion of energy reserves the ischemic brain critically compromises its ability to maintain ATP-dependent cellular membrane potentials, possibly resulting in an influx of sodium and calcium ions through voltage-gated ion channels leading to the demise of the cell through a cascade of excitotoxic cellular events [20]. Effectively, the reduction of seizure activity

following brain ischemia may be a crucial target for neuroprotection, and a rational complement to any neuroprotection strategy.

Most therapeutic sodium channel blocking compounds are use-dependent and only significantly block channels in the inactivated state. The result is that low frequency or basal levels of sodium channel function is left relatively unaffected, reducing toxicity, while high frequency stimulation (i.e. seizure activity) is attenuated [30]. Due to the membrane stabilizing properties of sodium channel blocking compounds they have also been studied as a possible therapies to relieve the excitotoxic effects of cerebral ischemia [34]. In the current study, we have evaluated the effects of the novel use-dependent sodium channel blocker RS100642 [9] to not only reduce brain injury following focal ischemia, but additionally to block ischemia-induced brain seizures.

2. Materials and Methods

2.1. Surgical Procedures including MCAo.

Male Sprague-Dawley rats (270-330g; Charles River Labs, Raleigh, VA) were used in all of the following procedures. Anesthesia was induced by 5% halothane and maintained at 2% halothane delivered in oxygen. Indwelling intravenous (i.v.) cannulas (PE-50) were placed into the left jugular vein of all animals for drug delivery. Depending on the experiment (see below), either 2 or 10 epidural electrodes (stainless steel screw electrodes, 0-80 x 1/8 in) were permanently implanted and fixed to the skull using dental acrylate cement [33]. Body temperature was maintained normothermic (37 ± 1°C) throughout all surgical procedures by means of a homeothermic heating system (Harvard Apparatus, South Natick, MA). Food and water were provided "ad libitum" pre- and post-surgery and the animals were individually housed under a 12 h light/dark cycle. The facilities in which the animals were housed were maintained and fully accredited by the American Association of Laboratory Animal Care. In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council.

Three to five days following the surgical procedures described above the rats were re-anesthetized and prepared for temporary focal ischemia using the filament method of middle cerebral artery occlusion (MCAo) and reperfusion as described elsewhere [31]. Briefly, the right external carotid artery was isolated and its branches coagulated. A 3-0 uncoated monofilament nylon suture with rounded tip was introduced into the internal carotid artery via the external carotid artery and advanced (approximately 20 mm from

the carotid bifurcation) until a slight resistance was observed, thus occluding the origin of the middle cerebral artery (MCA). The endovascular suture remained in place for 1 or 2 h and then retracted to allow reperfusion of blood to the MCA. Following MCAo surgery, animals were placed in recovery cages with ambient temperature maintained at 22°C.

2.2. Neuroprotection dose response (2h MCAo / 22h recovery)

Two cortical electrodes were placed over the right (injured) parietal cortex (4mm right, 4mm right and 4mm posterior to bregma). Rectal temperatures were monitored pre-MCAo and 0.5, 2, 4, 6, 24 h post-MCAo. EEG samples were recorded in the *anesthetized* animal pre-MCAo, during reperfusion and again at 24 h. I.v. injections of either vehicle or RS100642 (0.01-5.0 mg/kg) were give at 0.5, 2, 4, and 6 h post-MCAo. EEG power was calculated from 2 min EEG samples using QND EEG analysis software (Neurodata, Pasadena, CA). Power values at 2 h were compared to baseline, pre-MCAo values to obtain the percent drop in power. Percent recovery of EEG power was calculated as the increase at 24 h compared to the drop at 2 h. If the animals did not show at least an 80% drop in EEG amplitude immediately prior to reperfusion they were excluded from the study. Neurological evaluations (see below) were scored pre-MCAo, immediately before reperfusion at 2 h, and again at 24 h. At 24 h post-MCAo final EEG and neurological measures were taken following which animals were euthanized and brains collected for infarct analysis.

2.3. Brain seizures / long-term recovery (1h MCAo / 70 h recovery)

Ten cortical electrodes were permanently fixed to the rat skull, located as shown in figure 1. Animals were housed in custom designed plexiglas recording chambers (Dragonfly Inc., Ridgeley, WV) equipped with multi-channel swivel commutators (Plastics One, Roanoke, VA). On the morning of the experiment, the rats were connected to the swivel system by flexible shielded cables providing a noise-free connection from the unrestrained rat to a Grass model 7D polygraph and digital analysis system using Harmonie software (Astro-Med, West Warwick, RI) while permitting freedom of movement by the animals during all phases of the experiment. EEG samples were recorded in *awake, unanesthetized* animals pre-MCAo, during the 1 h MCA occlusion, and continuously for 5 h following reperfusion. Fifteen min EEG samples were again recorded at 24, 48, and 72 h post-MCAo. Ictal seizure events were defined as generalized epileptiform spike/slow-wave activity. Rectal temperatures were monitored pre-MCAo and 0.5, 2, 4, 6, 24, 48, and 72 h post-MCAo. I.v. injections of vehicle or RS100642 (1.0mg/kg) were administered either at 0.5, 2, 4, and 6 h post-MCAo or 0.5 h pre-MCAo and 2, 4, and 6 h post-MCAo. Neurological evaluations (see below) were scored pre-MCAo, immediately before reperfusion at 1 h, and again at 24, 48, and 72 h. At 72 h post-MCAo the animals were euthanized and brains collected for infarct analysis.

2.4. Neurological testing

Neurological scores (NS) were derived using a 10 point sliding scale. Each animal was examined for reduced resistance to lateral push (score = 4), open field circling (score = 3), and shoulder adduction (score = 2) or contralateral forelimb flexion (score = 1)

when held by the tail (modified from [4]). Rats extending both forelimbs toward the floor and not showing any other signs of neurological impairment were scored 0. Using this procedure, maximal neurological severity was measured as an NS = 10. In the present study, all rats subjected to MCAo either exhibited a neurological score of 10 when examined 1 or 2 h post-occlusion (i.e. immediately prior to reperfusion) or they were dropped from the study.

2.5. Infarct analysis.

From each rat brain, analysis of ischemic cerebral damage included total and core infarct volumes and hemispheric infarct size (calculated as percentage of total hemispheric volume, to exclude the possible contributing effect of hemispheric edema to infarct size). Infarcted regions were evaluated using TTC (2,3,5-triphenyl tetrazolium chloride) staining from seven coronal sections (2 mm thick) taken from the region beginning 1 mm from the frontal pole and ending just rostral to the cortico-cerebellar junction. Core injury was defined as brain tissue completely lacking TTC-staining while total injury was specified as all ipsilateral tissue showing a loss of stain as compared to the contralateral, uninjured hemisphere. Computer-assisted image analysis was used to calculate infarct volumes and has been described in detail elsewhere [31]. Briefly, the posterior surface of each TTC stained forebrain section was digitally imaged (Loats Associates, Westminster, MD) and quantified for areas (mm^2) of ischemic damage. Sequential integration of the respective areas yielded total and core infarct volumes (mm^3). Similarly, ipsilateral and contralateral hemispheric volumes were measured

where hemispheric swelling (edema) was expressed as the percent increase in size of the ipsilateral (occluded) hemisphere over the contralateral (uninjured) hemisphere.

2.6. Data analysis.

Data are presented as the mean ± standard error of the mean. Unless otherwise noted, statistical analysis of neuroprotective recovery was done by ANOVA followed by Dunnet's post-hoc test to compare individual treatment doses to the vehicle, control group. Statistical analyses were calculated using Minitab Statistical Analysis software program. For the neuroprotection ED_{50} the criteria for identifying a positive responder was defined as previously described [32]. Potency and ED_{50} calculations were performed using the Pharmacological Calculations Computer Programs [29].

2.7. Compounds.

RS100642 and Mexiletine were received from Roche Biosciences (Palo Alto, CA). The compounds were dissolved in a vehicle of distilled, deionized water immediately prior to testing and administered in a volume of 1ml/kg of body weight.

3. Results.

3.1. MCAo Injury.

Control vehicle-treated rats subjected to MCAo/reperfusion injury and either 24 or 72 h of recovery exhibited striatal and cortical brain infarction in the right hemisphere from approximately 3 to 11 mm from the frontal pole (fig. 2). Two hours of MCAo followed by 24 h of recovery resulted in total infarct volume in control rats of 378 ± 12 mm^3 and core infarct volume of 220 ± 16 mm^3 with the total infarct representing approximately 36%, and the core infarct approximately 21%, hemispheric infarction. One hour of MCAo followed by 72 h of recovery resulted in a total infarct volume in control rats of 284 ± 12 mm^3 and core infarct volume was 140 ± 19 mm^3 with the total representing 32%, and the core 16%, hemispheric infarction. MCAo resulted in significant hemispheric edema representing approximately an 8% and 6% increase in cerebral volume compared to the contralateral, uninjured hemisphere for the 24 and 72 h endpoints, respectively. Neurological function, which was severely impaired at 1 and 2 h post-MCAo (NS = 10 ± 0), exhibited a significant degree of spontaneous recovery (NS = 7.8 ± 1.1 at 24 h and NS = 2.2 ± 0.5 at 72 h). However, none of the injured vehicle-treated animals completely recovered neurological function (NS = 0), with at least contralateral forelimb flexion and shoulder adduction (NS = 3) still evident in all rats examined at 24 h, and at least contralateral forelimb flexion (NS =1) evident in all rats at 72 h post-injury.

All injured animals lost approximately 13-18% body weight over the 24 h recovery period and a 16-23% loss at 72 h, regardless of treatment group, with no significant differences in body weight loss between groups. In vehicle-treated control

animals, MCAo caused a transient, mild hyperthermia (38.1 ± 1.0°C) that began to return to normal values (36.7 ± 0.7°C pre-occlusion) by 6 h post-occlusion (37.5 ± 0.7°C). At 24 h temperatures in control injured animals returned to normal and at 48-72 h exhibited a mild, albeit non-significant, drop in body temperature (36.7 ± 0.7°C, 35.6 ± 0.4°C and 35.7 ± 0.6°C, respectively), which was similar to our earlier findings[31,36]. At all doses and time-points, temperature measurements from RS100642 treated animals were not significantly different from the corresponding control, vehicle-treated animals.

3.2. Neuroprotection dose response (2h MCAo / 24 h recovery)

Post-treatment with RS100642 (administered i.v. starting a 0.5 h post-occlusion) significantly reduced ischemic infarction throughout the brain measured at 24 h post-occlusion (fig.2). A dose-dependent decrease in infarct volume was seen in both total and core infarction (fig.3) corresponding to a neuroprotection in these regions of 57 ± 10% and 64 ± 12%, respectively at the maximal efficacious dose (1.0 mg/kg), which corresponds to 16% and 8% hemispheric infarction in total and core regions respectively. The neuroprotection ED_{50} (and 95% confidence limits) based on reduction of core infarction was 0.026 [0.021-0.31] mg/kg. The effects of RS100642 to alter hemispheric swelling due to edema following MCAo were not statistically significant.

A progressive improvement in both EEG power and neurological score was seen with RS100642 treatment at 24 h post-occlusion as compared to the vehicle-treated group (fig 4A and 4B). At the 1.0 mg/kg dose this recovery corresponded to a 24% increase in EEG power over vehicle-treated animals and an improved neurological score of 2.1 ± 0.8 as compared to 7.8 ± 1.1 for the vehicle-treated group. One RS100642 animal showed

complete recovery (NS=0) and 4 animals only showed signs of contralateral forelimb flexion (NS=1)

Post-injury treatment with mexiletine (fig 5) resulted in a 37 ± 14% reduction of total, and 53 ± 16% reduction of core, infarction which corresponds to 24% and 10% hemispheric infarction in total and core regions respectively. This recovery also corresponded to a 28 % increase in EEG power over vehicle-treated animals and an improved neurological score of 3.3 ± 1.2 as compared to 7.8 ± 1.1 of the vehicle-treated group. No significant effect on cerebral edema was measured as compared to the vehicle group. Critically, testing higher doses of mexiletine (i.e. 20 mg/kg) resulted in convulsant seizures in two animals and therefore further studies with mexiletine were halted.

3.3. Long term recovery (1 h MCAo / 72 h recovery)

Both pre- and post-MCAo treatment with RS100642 (1.0 mg/kg) resulted in a significant decrease in cerebral infarction when evaluated after 72 h of MCAo/reperfusion (fig.6A). The reduction of total and core infarct volumes corresponded to 51 ± 14% and 60 ± 18% (pre-treatment group) and 46 ± 12% and 65 ± 14% (post-treatment group), respectively. Similarly, the overall percent hemispheric infarction was significantly reduced (total = 16%, core = 6%, pre-treatment) and (total = 17%, core = 5%, post-treatment). No significant effect on cerebral edema was measured as compared to the vehicle group. For the pre- and post-treated groups there was a significant improvement in neurological recovery at 72 h post-MCAo, NS = 0.6 ± 0.2 and

0.5 ± 0.3, respectively (fig. 6B) where two animals in each group exhibited complete recovery of neurological function (NS=0).

3.4. Ischemia-induced brain seizure activity

MCAo-injured animals exhibited non-convulsant seizures (NCS), predominately during the first hour of injury, which were attenuated following reperfusion of blood to the brain (as observed from EEG recordings). However, no behavioral convulsions were observed during brain seizure activity and in general animals were conscious and ambulatory, although occasionally wet dog shake (WDS) behavior was observed during NCS. The NCS were generalized spike/slow-wave complexes occurring at a frequency of 1-2 per second and lasting 134 ± 33 s with 4.2 ± 1.2 ictal events occurring in 4 out of 5 vehicle-treated animals (fig. 7). On average, NCS began 26 ± 6 min following occlusion of the MCA in vehicle-treated animals. Furthermore, NCS were generalized to the entire brain and were recorded from all 10 cortical electrodes. Each consecutive seizure spike gradually increased in amplitude (fig. 7) and following resolution of seizure activity a period of post-ictal EEG depression occurred. Pre-treatment with RS100642 (1.0 mg/kg) completely blocked all NCS activity. With 30 min post-MCAo treatment of RS1000642 (1.0mg/kg), 3 of 4 animals experienced NCS but only 1 event occurred per animal and was shorter in duration (39 ± 12 s, $p<0.05$, independent t-test as compared to vehicle group).

4. Discussion.

RS100642, a new sodium channel blocker and neuroprotective agent, is an analog of the use-dependent sodium channel blocker mexiletine [9]. This study describes the *in vivo* dose-dependent neuroprotection profile of post-injury treatment with RS100642 to reduce brain injury and improve functional recovery from experimental transient focal ischemia and reperfusion in the rat, as well as block ischemia-induced brain seizure activity. As noted above, previous *in vitro* studies with RS100642 have reported complete neuroprotection against injuries caused by hypoxia/hypoglycemia (i.e. ischemic injury) or veratridine, but not glutamate, in primary neuronal cultures [9] indicating a selectivity for sodium channels. RS100642 was also reported to possess sodium channel binding properties similar to mexiletine (pKi = 5.09 μM) and exhibit sodium channel binding in a use-dependent manner as determined by rat vagus nerve preparations [9]. In rodent models of focal ischemia sodium channel modulation with various sodium channel blockers including mexiletine, lamotrigine and BW619C89, has been described as neuroprotective [12,15,28] indicating the potential for targeting neuronal sodium channels as a neuroprotection mechanism to treat ischemic brain injury.

Although the brain only comprises 2% of the total human body weight it consumes 25% of the available glucose and 20% of systemic oxygen. Nearly half of the central nervous systems energy supplies are utilized to drive Na^+-K^+-ATPase ion pumps, which repolarize neurons following an action potential as well as maintain ionic balance in glial cells [18]. A complex series of events occurs when cells lose their source of glucose and oxygen following ischemia. One major consequence is that mitochondria are unable to produce the ATP needed by the ionic pumps [34]. Glial cells then become

unable to absorb extracellular glutamate, a process dependent on the Na^+ gradient created by Na^+-K^+-ATPase pumps, leading to excitotoxicity or overstimulation of the post-synaptic neuron. To further exacerbate the problem of increased excitation, the postsynaptic neurons become unable to pump out the accumulated intracellular Na^+ and cannot reestablish ionic gradients. The result is the necrotic bursting of cells due to osmotic swelling [8]. Even for surviving cells an increase in intracellular Ca^{++} levels, due to opening of Ca^{++} permeable ion channels and reverse operation of the Na^+/Ca^{++} exchanger, leads to a cascade of cellular death mechanisms involving delayed Ca^{++}-mediated apoptotic processes of cellular degradation [20].

In this study we have used a model of temporary MCAo and reperfusion to produce brain injury in the rat closely resembling clinical stroke pathology [26]. Histological evaluation of injured tissues following MCAo using TTC staining revealed brain regions to be either completely infarcted (those areas completely lacking TTC stain) or pathologically compromised (regions of light pink staining). TTC is reduced to a red-formazon product in normal brain tissue due to the presence of active mitochondrial enzymes, and has been shown to correlate to other common histological markers of brain injury [3,23]. This model of *in vivo* transient MCAo has been widely used as an experimental model of ischemic brain injury and has proven to be highly sensitive to various neuroprotective drug interventions [7,13,19,25,31,36,40]. In this study, post-injury treatment with RS100642 dose-dependently reduced brain infarct volume, increased recovery in EEG power over the injured cortex and showed improvement of neurological recovery as evaluated 24 h post-injury. The correlation of increased EEG recovery with a reduction of brain infarction is similar to the effects seen with other

neuroprotective compounds in this model, including a high affinity N-methyl-D-aspartate antagonist and an experimental anti-inflammatory agent [25,36].

Although the temporal pathobiology of brain injury may continue for several weeks, by 72 h the injury has reached maximum hemispheric infarction volume [16]. As such, the neuroprotective effects of RS100642 were also confirmed following longer recovery periods of 72 h. Importantly, the neuroprotective effects of RS100642 were not likely due to drug-induced brain hypothermia since rectal temperatures, which have been shown to correlate to brain temperatures [38,41], never fell below normal levels following MCAo and treatment with RS100642.

Coincident with the neuroprotective alleviation of brain injury following RS100642 treatment we measured a significant and profound reduction of brain seizure activity. Initiation of NCS following MCAo injury generally began within 30 min of MCAo. Although pre-ictal treatment with RS100642 completely blocked this activity, post-ictal treatment was also able to reduce seizure activity and still provide a significant and comparable neuroprotective effect. Previously, we have described the spatio-temporal pathophysiology of brain ischemia in the MCAo model using high resolution 10-electrode topographic mapping in the rat [17,37]. These studies revealed the presence of ischemia-induced brain seizures as well as significant disruption of the EEG activity throughout the injured brain. Although the direct relation between these types of seizures and their possible involvement in promoting the pathology of the brain injury has not been firmly established, this seizure activity is undoubtedly an inherent part of the overall pathophysiology of ischemic brain injury and likely detrimental to recovery. Sustained or repeated Na^+ channel openings has been shown to induce persistent Na^+ conductance

across cellular membranes likely due to a shift in Na^+ channel gating properties, reducing the likelihood of Na^+ channel inactivation [1,6]. Persistent Na^+ conductance can induce long-lasting depolarizing plateau potentials and has been proposed to underlie the presence of repeated depolarizing waves associated with seizures, spreading depression, as well as the sustained depolarizations of ischemic brain tissues [30]. Following brain injury, the modulation of sodium channels to reduce cellular depolarizations not only ameliorates the mechanisms of glutamate-induced excitotoxicity but can also maintain cellular membrane potentials by reducing activity of Na/K pumps (the major source of neuronal energy use) and in effect reduce the load on the already compromised energy reserves. Clinically, the therapeutic use of anticonvulsants following brain injury in not widely practiced except in those patients who develop convulsive seizure activity [2].

Sodium channels exhibit different electrophysiological properties including the classical fast inactivating, tetrodotoxin (TTX)-sensitive channels of α-subunits I, II, IIA, III, and PN1, slow inactivating, TTX-resistant α-subunits PN3 and NaN, and others including Na6 and NaG [10,11,22]. Each of these subunits also exhibits different patterns of expression throughout the central and peripheral nervous system [11]. Furthermore, it has been implicated that down-regulation of sodium channels during hypoxia may be a survival strategy of diving turtles, whose brains are deprived of oxygen for extended periods of time [24]. Our lab is currently evaluating the temporal profile of sodium channel expression in the MCAo model to determine the optimal time course of intervention as well as specific sodium channel subunits to target in neuroprotective drug development [39].

Treatment with RS100642 also provided an improved safety profile (i.e. neuroprotective to neurotoxic index) as compared to mexiletine. Mexiletine evaluation in this study was limited by its effect to induce seizure toxicity at a dose (20 mg/kg), or only twice that of its determined neuroprotective dose (10 mg/kg). Not only was RS100642 more efficacious and more potent than mexiletine in reducing brain infarction and improving functional recovery, it produced no signs of neurotoxicity in the neuroprotection dose range. Importantly, separate experiments in normal rats given acute subcutaneous injections of RS100642 up to 600 mg/kg also provided no evidence of brain seizure activity (unpublished observations). Other clinically available compounds with sodium channel blocking and neuroprotection properties have been associated with cardiovascular effects at higher doses [28]. For example, the sodium channel blocker lamotrigine, which provided a 38% cortical neuroprotection (8mg/kg, i.v.) in MCAo injured rats, also caused decreases in blood pressure at higher doses (50 mg/kg, i.v.) [28].

In conclusion, a comprehensive dose-response study has established the neuroprotective efficacy of RS100642 to effectively reduce brain infarct volume and improve functional recovery following post-injury treatment in a focal cerebral brain injury model of stroke. Furthermore, this neuroprotective effect was associated with an attenuation of ischemia-induced seizure activity. Collectively, these results support the possible clinical efficacy of RS100642 as a post-injury treatment of ischemic type brain injury. Further studies with this compound will be aimed at evaluating long term functional recovery using high resolution EEG mapping and defining the "therapeutic window" of treatment by delaying time of initial treatment post-injury.

Figure Titles

Figure 1. Location of cortical EEG electrodes on the rat skull.

Figure 2. Representative coronal brain sections stained with TTC from vehicle and RS100642-treated animals following 2h MCAo and 22 h reperfusion and recovery.

Figure 3. Dose response effect of RS100642 to decrease brain infarct volume following 2h MCAo and 22 h reperfusion and recovery (n = 6-8 per group). *P<0.05, **P<0.01, ANOVA followed by Dunnet's post hoc analysis.

Figure 4. Effect of RS100642-treatment following 2 h of MCAo and 22 h recovery and reperfusion to A) increase recovery of EEG power *P<0.05, ANOVA followed by Dunnet's post hoc analysis, B) improve neurological outcome *P<0.05, **P<0.01, Mann-Whitney U test.

Figure 5. Effects of maximally efficacious doses of RS100642 and mexiletine to reduce total (A) and core (B) brain infarct volume following 2h MCAo and 22 h reperfusion and recovery. *P<0.05, **P<0.001, ANOVA followed by Dunnet's post hoc analysis.

Figure 6. Effect of 30 min pre- or 30 min post-MCAo treatment with RS100642 (n = 4-5 per group) to A) reduce brain infarct volume *P<0.05, ANOVA followed by Dunnet's post hoc analysis, and B) improve neurological outcome *P<0.05, Mann-Whitney U test, following 1h MCAo and 70 h reperfusion and recovery.

Figure 7. Effect of 30 min pre- or 30 min post-MCAo treatment with RS100642 to reduce the number of ictal seizure events. The number of animals exhibiting seizure activity are given in parentheses. Baseline and ictal seizure activity are shown with vertical bars representing 100μV and the horizontal bar representing 1 min of EEG recording.

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Figure 1

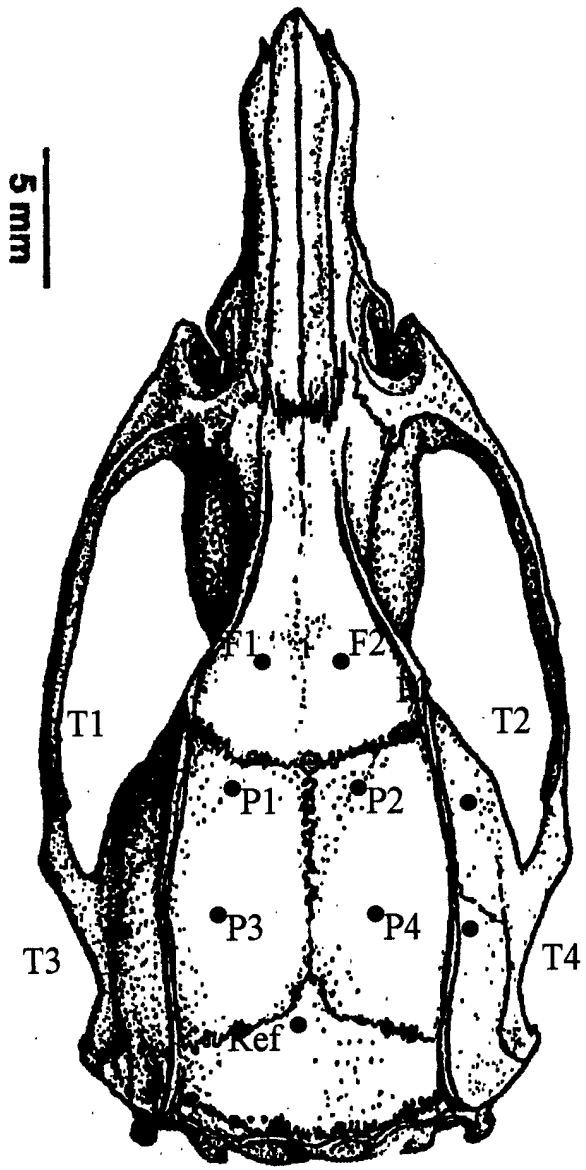


Figure 2

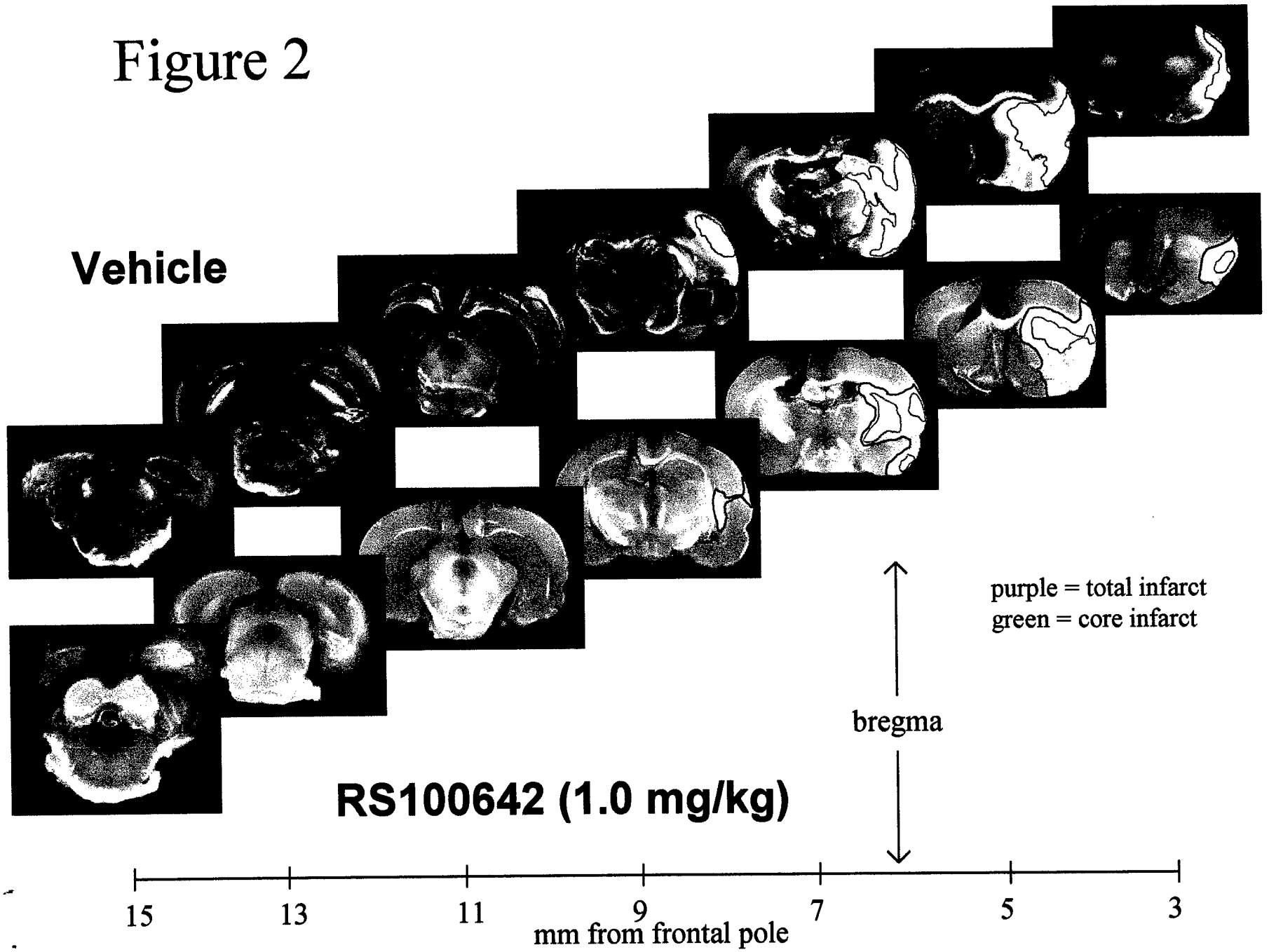

Vehicle
RS100642 (1.0 mg/kg)
purple = total infarct
green = core infarct
bregma
15
13
11
9
7
5
3
mm from frontal pole

Figure 3

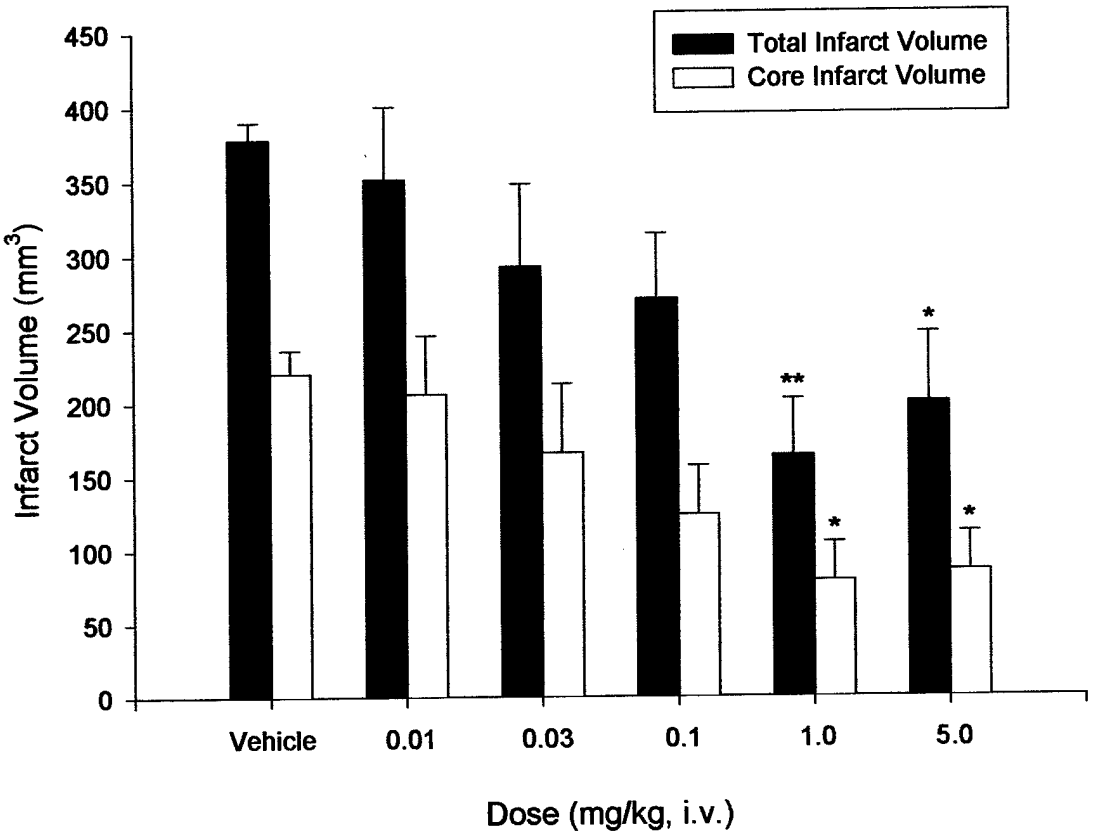
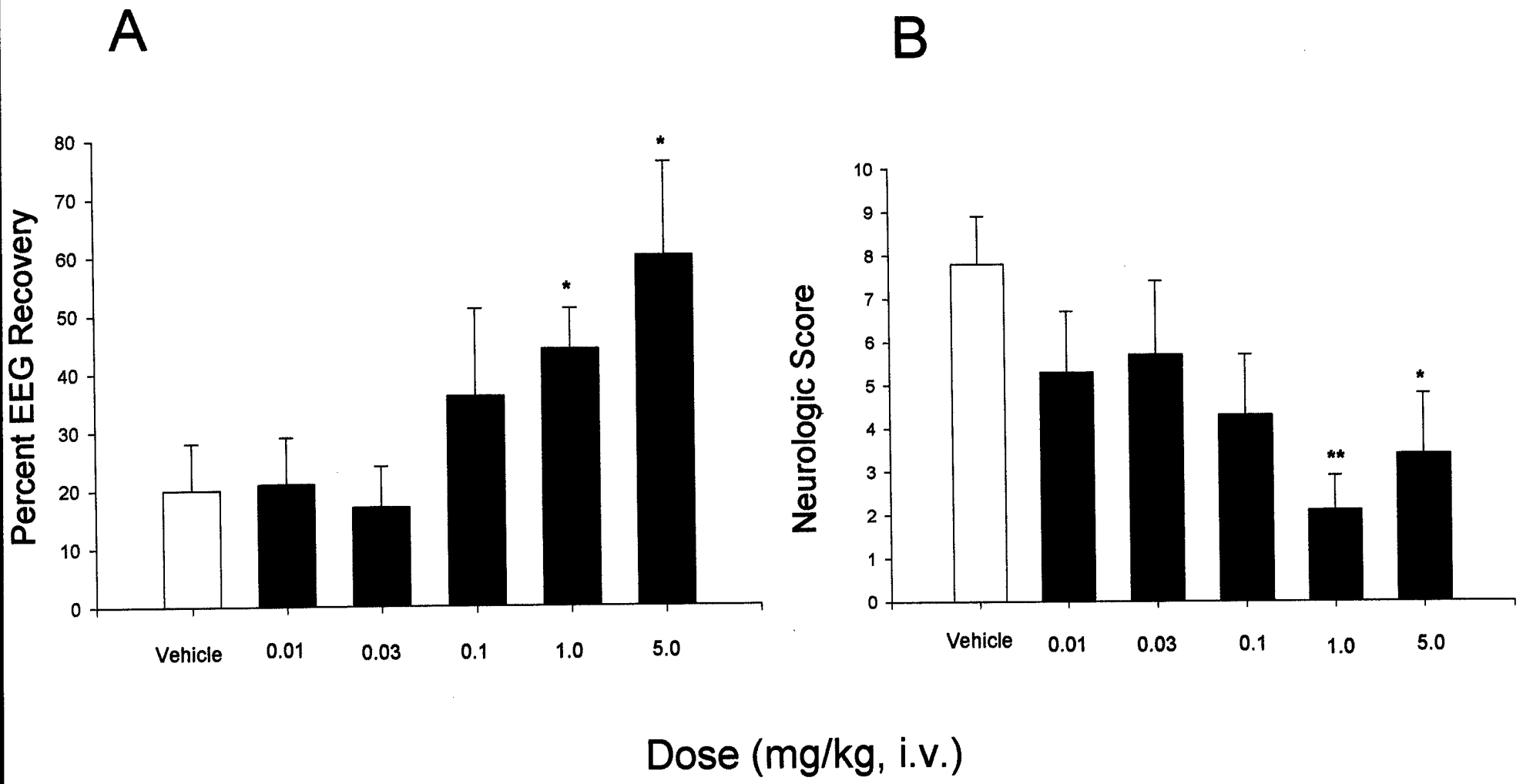

Total Infarct Volume
Core Infarct Volume
Infarct Volume (mm3)
450
400
350
300
250
200
150
100
50
0
**
*
*
Vehicle
0.01
0.03
0.1
1.0
5.0
Dose (mg/kg, i.v.)

Figure 4


A
B
Percent EEG Recovery
Neurologic Score
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10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
8
9
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*
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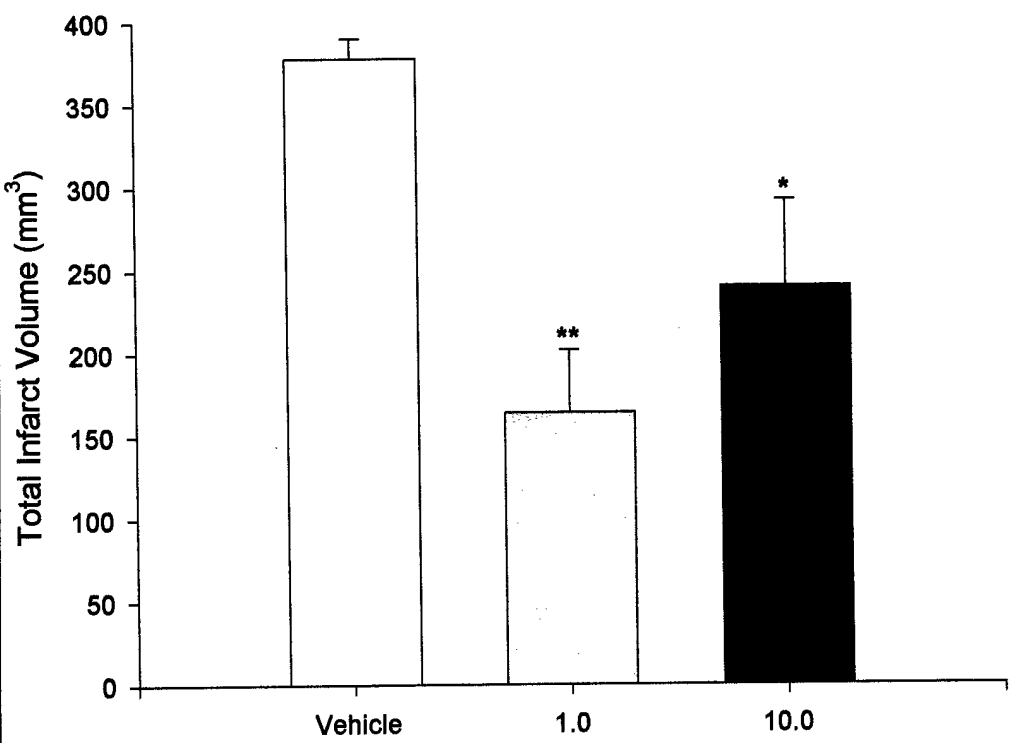
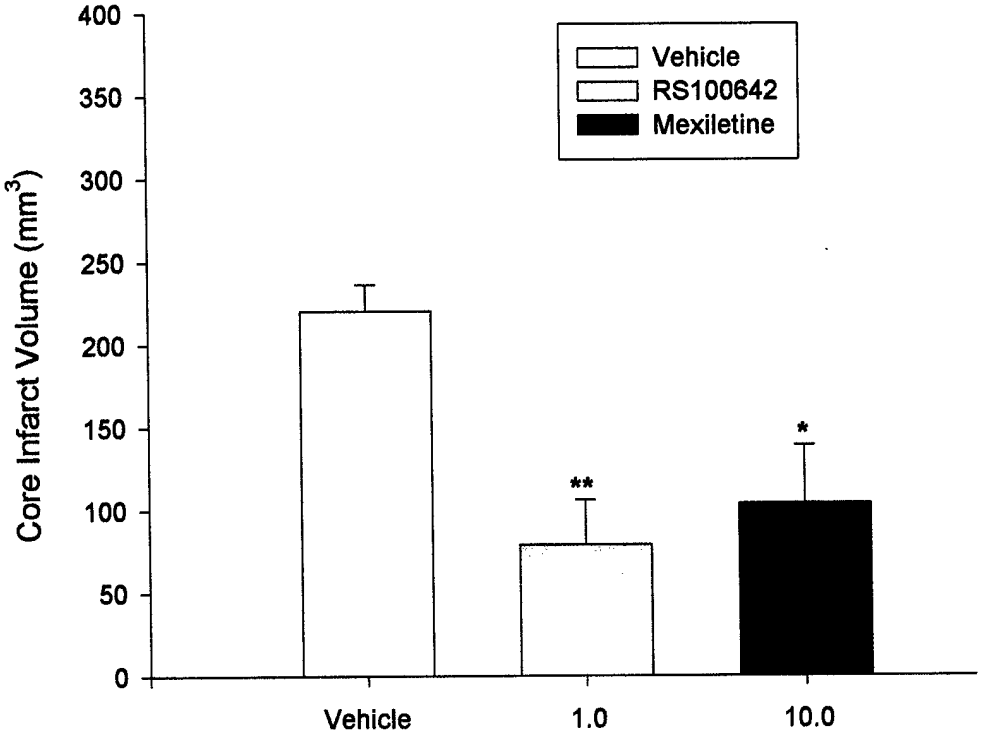
Vehicle
0.01
0.03
0.1
1.0
5.0
Dose (mg/kg, i.v.)

Figure 5





Dose (mg/kg, i.v.)

Figure 6

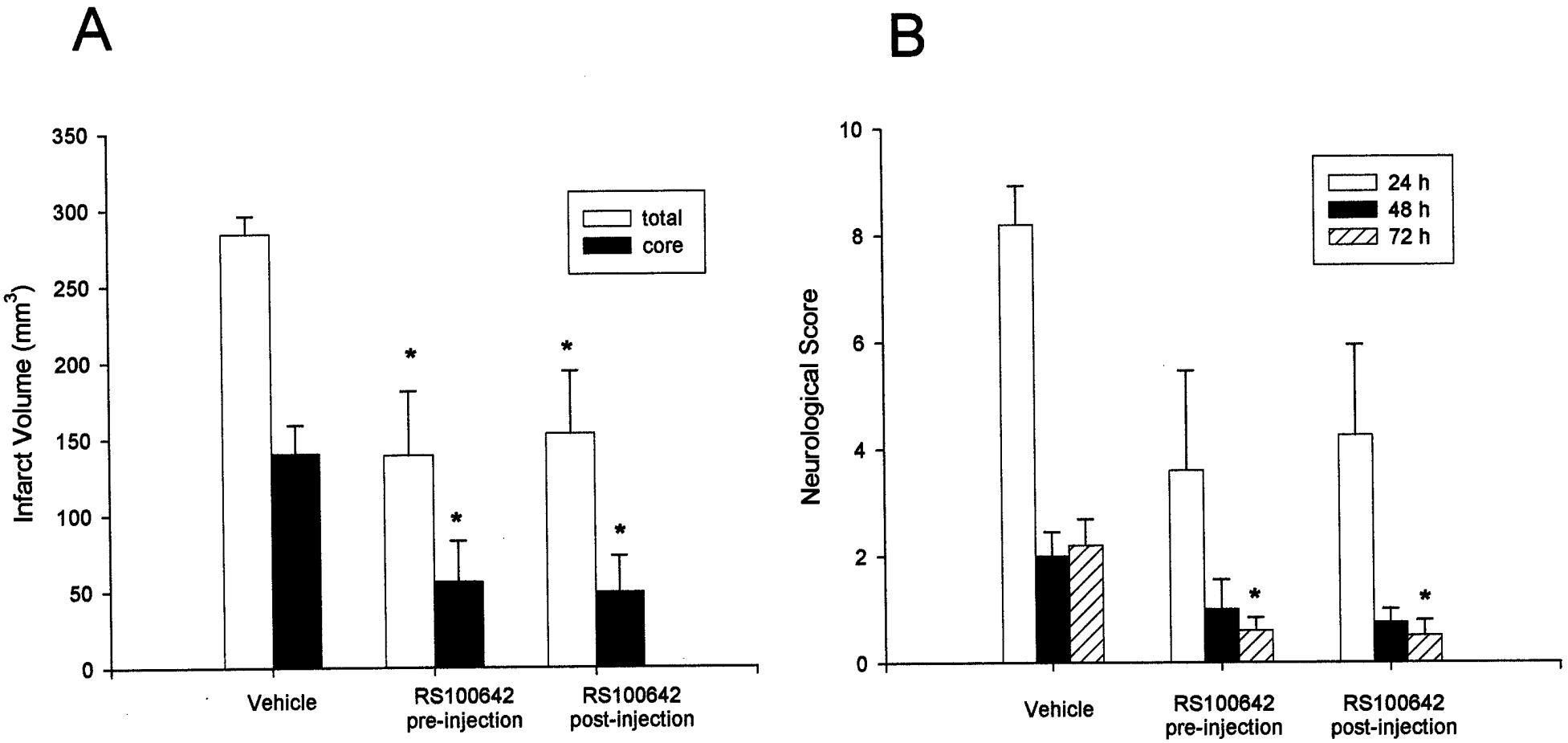
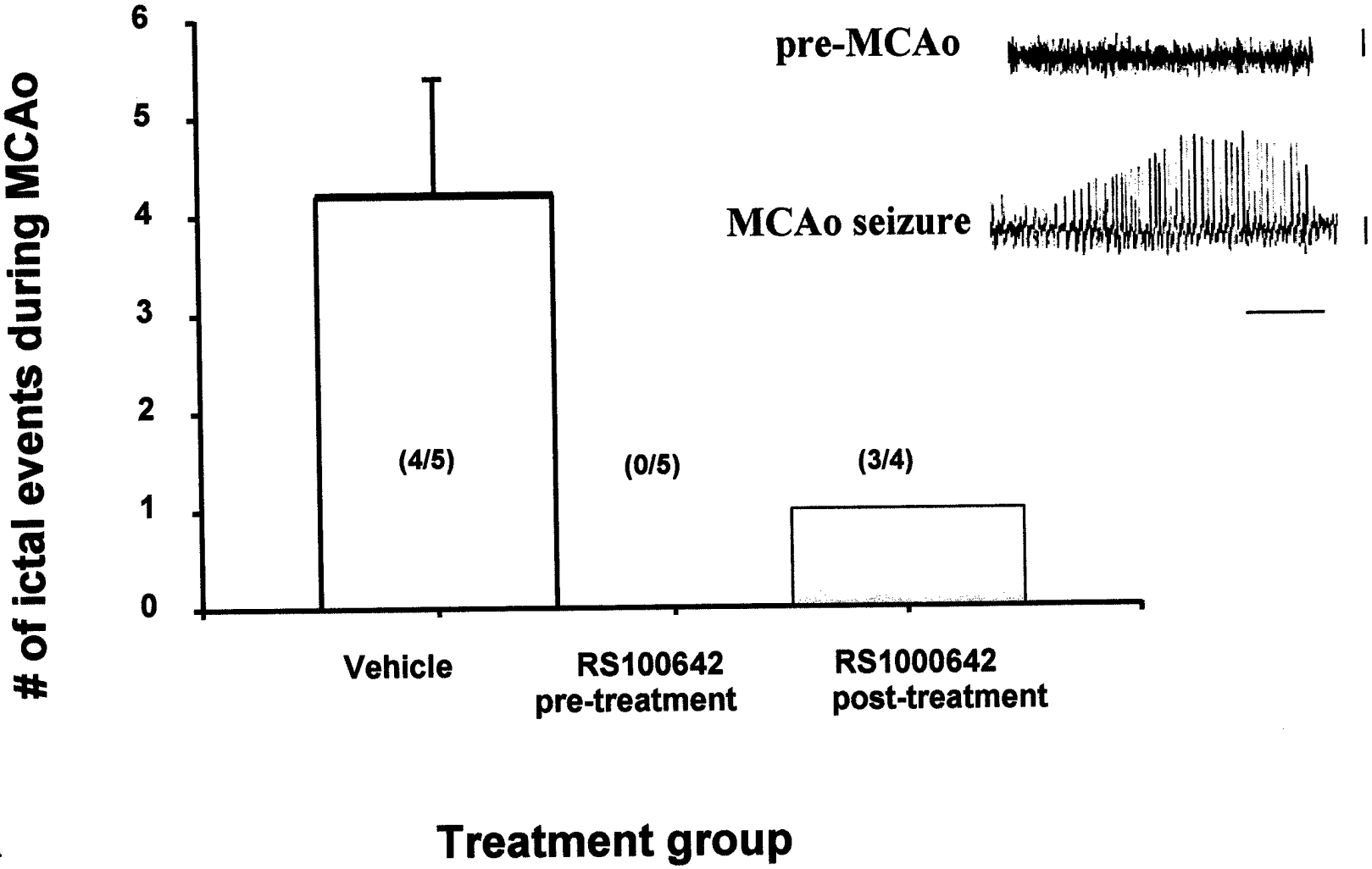

A
B
Infarct Volume (mm3)
350
300
250
200
150
100
50
0
total
core
*
Vehicle
RS100642
pre-injection
RS100642
post-injection
Neurological Score
10
8
6
4
2
0
24 h
48 h
72 h

Figure 7


pre-MCAo
MCAo seizure
# of ictal events during MCAo
6
5
4
3
2
1
0
(4/5)
(0/5)
(3/4)
Vehicle
RS100642
pre-treatment
RS1000642
post-treatment
Treatment group

Soc. Neuroscience Abst. : Oct. 2001

EXPRESSION OF SODIUM CHANNEL GENES FOLLOWING ISCHEMIC INJURY: AN *IN SITU* HYBRIDIZATION STUDY. A. J. Williams[1], F. C. Tortella[1]*, C. Yao[1], Z. Y. Yu[3], S. L. Hale[2], R. Berti[1] and J. R. Dave[1]. [1]Div. of Neurosci. and [2]Div. of Pathol., Walter Reed Army Inst. Res. and [3]Naval Medical Res. Ctr., Silver Spring, MD 20910.

Voltage-dependent sodium channels (NaChs) can modulate neuronal excitability and are considered primary targets of several neuroprotective drugs. We have previously reported (Yao et al., Neurosci. Abstr. 26, 2000) the expression of mRNA for the NaCh genes rBI, rBIII, PN1 and PN3 in normal and ischemic rat brain by quantitative RT-PCR where the relative abundance of NaCh mRNAs in normal rat brain was rBI>PN3>rBIII>PN1, and the rBI gene exhibited a significant down-regulation in the ischemic hemisphere measured form 6-48 h post-injury. The objective of this study was to determine the differential and anatomical distribution of these NaCh mRNAs following brain injury produced by middle cerebral artery occlusion (MCAo) using *in situ* hybridization techniques. Preliminary results have revealed a low hybridization signal of rBI mRNA not only in the ischemic region but also in the area of the contralateral hemisphere proximal to the ischemic region. This observation was unexpected since that part of the contralateral hemisphere exhibiting lower rBI mRNA hybridization appeared normal when stained with 2,3,5-triphenyltetrazolium chloride (TTC). Additional *in situ* studies are currently in progress to determine the expression profile of other NaCh (rBIII, PN1 and PN3) genes following MCAo. These findings begin to define the anatomical distribution of NaCh gene expression in rat brain, and provide further information on their possible role in the cellular mechanisms of CNS injury. (DoD Funding)